The cellular and molecular properties of genistein against *C. albicans* infection

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- **Cui, S.,** Jiang, JJ., Ai, H., Li, J., Bilitewski, U., Niu, JZ. (2010) Effect of genistein on the proliferation, morphology and cell cycle of macrophage RAW264.7. *Beijing Journal of Traditional Chinese Medicine*, 39 (3): 40-43.
- **Cui, S.,** Jiang, JJ., Li, Y., Bilitewski, U., Niu, JZ. (2010) Effect of genistein on the LPS induced macrophage function. *Acta Chinese Medicine and Pharmacology*, 38(2):13-16.

Conferences

- **Cui, S.,** Klippel, N., Bilitewski, U. (2010) Role of *C. albicans* MAPK cascade in the interaction with macrophages, Poster, 3rd Joint conference German Society for Hygiene and Microbiology (DGHM) and Association for General and Applied Microbiology (VAAM), Hannover.
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ABBREVIATIONS

ADP	Adenosindiphosphate
AIDS	Acquired immunodeficiency syndrome
AP-1	Activator protein 1
ATCC	American Type Culture Collection
ATP	Adenosintriphosphate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
C.albicans	*Candida albicans*
CAF2-1	*C.albicans* strain
CARD9	Caspase recruitment domain protein 9
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modification of Eagle's medium
DMSZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E.coli	*Escherichia coli*
EDTA	Ethylendiamine acetic acid
Em.	Emissions wavelength
ERK	Extracellular signal-regulated kinase
Ex.	Excitation wavelength
ELISA	Enzyme linked immunoabsorbent assay
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FMN	Flavin mononucleotide
GFP	Green fluorescent protein
GlcNAc	N-Acetylglucosamine
GM-CSF	Granulocyte colony-stimulating factor
HRP	Horseradish peroxidase

IFN-γ	Interferon gamma
IgG	Immunglobulin G
IL	Interleukin
IL-1Ra	Interleukin 1 receptorantagonist
iNOS	Inducible nitric oxide synthase
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulation factor
ITAM	Immunoreceptor tyrosine-based activation-like motif
IκB	Nucluear Factor κB-inhibitor protein
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
LR	C-type Lectin receptor
LRR	Leucin-rich repeat
MAL	TIR-domain-Adapterprotein
MAP	Mitogen activated protein
MIP	Macrophage inflammatory protein
MR	Mannosereceptor
MKP-1	MAP-Kinase-Phosphatase 1
MyD88	Myeloid differentiation primary response gene 88
NADPH	Nicotinamidadenindinukleotidphosphat
NFAT	Nuclear factor activated T-cells
NF-κB	Nuclear Factor kappa B
NK cells	Natural killer cells
NOS	Nitric oxide synthase
NO	Nitric oxide
OD	Optical Density
p38	Mitogen-activated protein kinase 38
PBS	Phosphate buffered saline
PDVF	Polyvinylidene fluoride
Pen	Penicillin
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol 3-kinases

PRR	Pattern-recognition receptor
PTK	Protein tyrosine kinase
RAW264.7	Macrophage cell line
RIP1	Receptor interacting protein
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT	Room temperature
rpm	Rounds per minute
S.cerevisiae	*Saccharomyces cerevisiae*
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Strep	Streptomycin
Syk	Spleen tyrosine kinase
TBK1	TANK-binding kinase
T_H	T-Help cell
Thr	Threonine
TIR	TLR-interleukin 1 receptor
TLR	Toll-like-Receptor
TD	Transmembrane domain
TMB	3,3´,5,5´-Tetramethylbenzidine
TNF	Tumor necrose factor
TRAF	TNF Receptor Associated Factor
TRAM	TIR-domain-containing adapter-inducing interferon-β-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
WST1	4-[3-(4-Jodophenyl)-2-(4-Nitrophenyl) -2H-5-Tetrazolio]-1,3-Benzoldisulfonate
YPD	Yeast extract peptone dextrose

1 ABSTRACT

C. albicans is a ubiquitous fungal organism that often colonizes the skin and the mucosal surfaces of healthy individuals, without causing disease. However, when the normal host defence mechanisms are impaired, *C. albicans* can become a pathogen. Candidaemia has an incidence of between 1.1 and 24 cases per 100,000 individuals and an associated mortality rate of more than 30%. Thus, the elucidation of the mechanisms of the interactions of *C. albicans* and the host is not only a highly relevant research topic, but also can lead to the identification of novel targets and promising compounds for treating *C. albicans* infections.

We chose as chemical compound the isoflavone genistein for our studies. Genistein is a well known natural compound which is present in soy foods and exerts many beneficial functions (anticancer, anti-inflammatory, antioxidant). However, until now little is known about the effects of genistein on *C. albicans*, the function of macrophages, and the interactions between both organisms. We used the murine macrophage cell line RAW264.7 as target cell line. We found that genistein could influence macrophage functions: at concentrations of 100 - 50 μM, genistein reduced the cell viability to 70 % - 80 % (after 24 h) and 50 % - 60 % (after 48h), which was due to G2/M phase cell cycle arrest. Treatment of the macrophages with genistein for 24 or 48 h also led to significant morphological changes, such as elongation of the cells and development of long pseudopodia-like protrusions. By staining the F-actin cytoskeleton, we observed accumulation of actin - filaments at the edges of the cells. Phagoyctosis is one of most important functions of macrophages. After treatment of the macrophages with genistein, the phagocytotic efficiency for *C. albicans* was decreased in a time- and dose-dependent manner. Moreover, the production of cytokines (TNF-α, IL-10) stimulated by *C. albicans* was strongly inhibited by genistein. Genistein also blocked the response of macrophages to bacterial stimulants, such as LPS, i.e. the secretion of NO, TNF-α, and IL-10, which is related to the inhibition of the activation of transcription factors NF-κB and AP-1.

Additionally, we investigated whether genistein could directly influence the pathogen, *C. albicans.* Our results showed that genistein blocked oxygen consumption of *C. albicans* and induced the production of reactive oxygen species. However, those effects were not observed in *S. cerevisiae. C. albicans* and *S. cerevisiae* significantly differ in the

composition of the respiratory chain, as in *S. cerevisiae* complex I is absent. From comparison with rotenone (specific complex I inhibitor) and antimycin A (specific complex III inhibitor), we concluded that complex I is the most likely target for genistein. Using DCIP as a mediator, we detected complex I reductase activity. Genistein did not act as an inhibitor of DCIP reduction by complex I as rotenone did. The results pointed out that genistein binds to complex I of *C. albicans* at a site different from that of rotenone.

Moreover, treatment of *C. albicans* with genistein enhanced the susceptibilty of the pathogen for phagocytosis by the macrophages and increased the production of the cytokines IL-10 and TNF-α. However, genistein did not influence the structure of the cell wall glucans and mannans of *C. albicans*, which was analysed by flow cytometry; whereas the cell surface hydrophobicity, which correlates with adherence, increased.

In conclusion, genistein could influence both the functions of host macrophages and the properties of the pathogen *C. albicans*. Thus, genistein may have antimycotic effects by acting on *C. albicans* to enhance the phagocytic activity of macrophages.

2 INTRODUCTION

Natural products, especially isoflavones, have attracted many attentions from scientists due to their natural origins and low side effects. Isoflavones are present in food, especially in soybean and some Chinese herbs. Their beneficial health effects are well studied, in particular with respect to cancer treatment.

With the widespread use of broad-spectrum antibiotics, spread of AIDS, in particular, immunosuppressive agents in therapy cancer treatment, the incidence of *C. albicans* infections increased year by year. Candidiasis accounted for 82 % of all fungal infections. Although prevention and treatment of *C. albicans* infections has made great progress, the mortality rate is still high. Due to the commensal nature of these pathogens, prevention of the outbreak of infections by the long term use of healthy food components is one of the promising and challenging research topics. Therefore, in these studies, we focus on the influence of the food constituent genistein on the co-existence of *C. albicans* and host cells, in particular macrophages.

2.1 Pharmacological activity of genistein

Flavonoids belong to a polyphenol subclass and are widely distributed in the plant kingdom. They are chemically categorized into different subtypes based on the connection of an aromatic ring to the heterocyclic ring as well as the oxidation state and functional groups of the heterocyclic ring (Tab. 2.1) [1]. Flavonoids are found in fruits, vegetables, legumes, herbs, spices, stems, flowers, as well as tea and red wine. They are consumed regularly in many countries in a healthy diet.

Natural flavonoids and isoflavonoids are currently of great interest due to their antioxidative and anticancer properties. Genistein (4’, 5, 7-trihydroxyisoflavone) (Fig. 2.1), a well studied isoflavone, is a major constituent of soyfoods and legumes such as lupin, fava beans, soybeans, kudzu, psoralea, and chickpeas. It shows a variety of biological effects, for example as a phytoestrogen or anti-cancer agent etc., but here we focus on the pharmacological activity related to microbial infections.

Tab. 2.1 Classification, prominent food flavonoids and typical food sources

Flavonoid class	Food flavonoid	Food source
Flavanols	Catechin, gallocatechin, epicatechin	Teas,red grapes and red wine
Flavanones	Naringenin, hesperetin, eriodictyol, taxifolin	Citrus foods
Flavones	Apigenin, luteolin, rutin chrysin	Green leafy spices
Isoflavones	Daidzein, **genistein**, glycitein, biochanin A, daidzin	Soybeans,soy foods and legumes
Flavonols	Kaempferol, myricetin, quercetin, isorhamnetin, tamarixetin	Nearly ubiquitous in foods
Anthocyanidins	Cyanidin, delphinidin, pelargonidin, apigenidin	Red, purple and blue berries

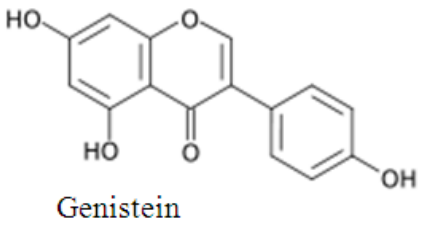


Fig. 2.1 Structure of genistein

2.1.1 Bioavailability of dietary genistein

Most flavonoids exist in plants as glycosidic conjugates, generally located in cell vacuoles. Bioavailability of these dietary components depends on relative uptake rates of conjugated and free forms, hydrolysis of glycosides by gut bacteria or gut wall enzymes, further metabolism such as glucuronides pathway within the liver, and excretion rates. The malonyl glucosides of daidzein and genistein found in soybeans are labile and are readily degraded to the non-acylated glucosides following cooking. The free aglycones, but not the glycosides, are absorbed from rat stomach. However, once in the small intestine, brush border lactase phlorizin hydrolase can effectively hydrolyze isoflavone glucosides. Nevertheless, in humans, isoflavones appear in blood plasma at a more rapid rate and at higher levels, following oral administration of aglycones as compared to glycosides. Moreover, genistein and daidzein, but not their glycosides, are readily transported across human intestinal epithelial cell monolayers [2].

2.1.2 Genistein as a tyrosine kinase inhibitor

Tyrosine kinase is an enzyme that transfers a phosphate group from high energy donor molecules such as ATP to tyrosine residues of proteins. It plays an important role in regulation signal transduction in human cells. Genistein was reported as a tyrosine kinase inhibitor by Akiyama in 1987 [3]. Genistein exerts anti-inflammatory and anti-infection effects due to this kinase inhibitiory property which will be described in the related paragraph.

2.1.3 Genistein as an antioxidant

Cells and tissues in the body are continuously threatened by damage caused by free radicals and reactive oxygen species, which are produced during normal oxygen metabolism or are induced by exogenous factors [4]. The increased production of reactive oxygen species accompany most forms of tissue injury, which have been implicated in a multitude of disease states ranging from inflammatory injury to myocardial infarction and cancer [5]. Genistein has been shown to protect cells against reactive oxygen species (ROS) by scavenging free radicals and reducing the expression of stress-response related genes [6, 7]. Genistein inhibits 12-O-tetradecanoylphorbol-13-acetate-induced hydrogen peroxide production in human polymorphonuclear leukocytes and HL-60 cells [8, 9]. The antioxidant mechanism which genistein shows leads to an inhibition of the activation of NF-κB stimulated by oxidative stress and chemokine-8 secretion, via a decrease in either caspase-3 or protein tyrosine kinase activity [10-13]. Thus, the ability of genistein to inhibit the generation of ROS, resulting in the inhibition of NF-κB activation, makes it a strong candidate as an antioxidant and a powerful chemopreventive agent.

2.1.4 Genistein and immunity

The effects of genistein in immunity are well studied among the isoflavones. In some studies, ovariectomized (OVX) mice were used to avoid the effect of endogenous estrogen. The results showed that the influence of genistein on OVX mice and on the non OVX model is different. Genistein induced dose-dependent reductions in thymic weight and size in OVX mice. It decreased thymocyte numbers by up to 86 % and reduced the number of peripheral lymphocytes and antigen-specific antibody titers [14, 15]. Treatment of genistein administered mice with anti-estrogen faslodex (ICI182780) partially restored thymic weight. Therefore, the effect of genistein could partly be related to interactions with the estrogen

receptor. Non-OVX mouse, daily feeded with 4-20 mg / kg genistein showed increases of non-specific immune functions in a dose-dependent manner. Also the production of IL-4 and IFN-γ was increased and cytotoxic T cell and NK cell activity was enhanced [16-18]. Meanwhile, *in vitro* experiments found that relatively high doses of genistein can inhibit mitogen- and alloantigen-induced lymphocyte proliferation [19].

2.1.5 Genistein and inflammation

Genistein possess anti-inflammatory properties as a result of different mechanisms (Tab. 2.2). It can inhibit phospholipase A2, cyclooxygenases, and lipooxygenases resulting in the reduction of levels of pro-inflammatory mediators. *In vivo*, genistein markedly attenuates ovalbumin-induced bronchoconstriction, pulmonary eosinophilia, and airway hyper-responsiveness in a guinea pig model of asthma. This anti-inflammatory effect may be mediated by the inhibition of the tyrosine kinase signalling cascade [20]. Intraperitoneally injected genistein was shown to protect rats from the endotoxin-induced organ failure [21], and later treatment with genistein reduced the degree of inflammation and joint destruction in collagen-induced arthritic mice. These therapeutic effects were mediated by a modulation of granulocytes, monocytes, and lymphocytes [22].

In vitro, genistein is able to suppress NO production in LPS activated murine macrophages in a dose-dependent manner by three mechanisms: scavenging of NO radicals, inhibition of inducible nitric oxid synthase (iNOS) enzyme activity, and inhibition of iNOS gene expression [22]. Genistein was shown to inhibit TNF-α production in LPS-induced RAW264.7 macrophages cells [23] and to inhibit IL-1β, IL-6, and TNF-α production from LPS-stimulated human blood monocytes [24]. The inhibitory effects of genistein on IL-6 production have been shown in different cells: cultured human intestinal cells Caco2 [25], osteoblast cells [26], human gastric epithelial cells [27], or macrophages [28].

Tab. 2.2 Anti-inflammatory mechanisms of genistein [29]

Activity	Mechanism	Effect
Antioxidant activity	Radical scavenging	Lower the amounts of free radicals
	Inhibition of ROS production	
	Inhibition of pro-oxidant enzymes	Inhibition of lipidic peroxidation
Modulation of inflammatory cells	Modulation of enzymatic activity	Inhibition of inflammatory cells activation
	Modulation of secretory processes	
Modulation of proinflammatory enzymes	Inhibition of arachidonic acid enzymes	Inhibition of the production of inflammatory mediators
	Inhibition of NO synthase	NO, leukotrienes, prostaglandins
Modulation of proinflammatory mediators	Modulation of cytokine production	Inhibition of the inflammatory cytokines: TNF-a and interleukines
Modulation of proinflammatory gene expression	Modulation of signal transduction	Inhibition of proinflammatory gene transcription

2.1.6 Genistein and infection

Genistein was reported to stimulate growth of *C. albicans* by increasing the rate of germination and enhancing Hsp90 expression. These biological effects may be attributable to genistein's activity as a phytoestrogen [30]. Meanwhile, genistein strongly inhibited *C. albicans* yeast adhesion to Vitronectin and human endothelial cells by blocking the phosphorylation of endothelial cell proteins, therefore, it significantly reduced endocytosis of *C. albicans* due to inhibition of tyrosine kinase [31, 32].

2.1.7 Genistein involved in signal transduction pathways

Genistein modulates several key elements of signal transduction pathways related to cellular growth and survival. These cover the full spectrum of the intracellular signalling network.

2.1.7.1 Inhibition of the activation of NF-κB

The transcription factor NF-κB plays an important role in the control of cell growth, differentiation, apoptosis, and stress-response. Under non-stimulating conditions, NF-κB is

sequestered in the cytoplasm through tight association with the impeding IκB proteins. Following stimulation, the IκB proteins are phosphorylated and degraded, allowing the NF-κB to translocate to the nucleus, bind to the NF-κB specific DNA binding sites or interact with other transcription factors, and thus regulate gene transcription. NF-κB plays a major role in pathways involved in inflammation and innate immune reponses [33].

Genistein blocks the activation of NF-κB concomitant with degradation of IκBα in human myeloid leukaemia cells [34]. Genistein inhibits the translocation of NF-κB to the nucleus, preventing NF-κB from binding to its target DNA and thereby inhibiting the transcription of NF-κB downstream genes. This process ultimately inhibits cell growth and also induces apoptotic cell death. Genistein has also been found to potentiate the antitumor activity of chemotherapeutic agents through regulation of NF-κB [35, 36] .

2.1.7.2 Regulation of Akt signalling pathway

Akt is a serine/threonine protein kinase that plays a key role in multiple cellular processes. Akt signalling is another important signal transduction pathway that plays a critical role in controlling the balance between cell survival and apoptosis [37]. Akt also regulates the NF-κB pathway via phosphorylation and activation of molecules in the NF-κB pathway [38, 39]. Genistein treatment reduces the level of the phosphorylation of the Akt protein compared to control cells, resulting in a dose-dependent induction of apoptosis of cells that display constitutively active Akt [40]. Genistein pre-treatment abrogated the activation of Akt by EGF-stimulated PC-3 prostate cancer cells [41], demonstrating that genistein inhibits the activation of Akt, which may result in the inhibition of survival signals ultimately leading to induction of apoptotic signals. Genistein exerts its inhibitory effects on NF-κB pathway through the Akt signalling pathway in cancer cells [25]. Genistein has been shown to exert inhibitory effect on Akt activation induced by estradiol in MCF-7 cells [42, 43]. Collectively, these results demonstrate that genistein exerts its inhibitory effect on NF-κB signalling through Akt pathway. Thus, abrogation of NF-κB and Akt signalling pathway by genistein may be one of the molecular mechanisms by which genistein inhibits inflammation and cancer cell growth, and induces apoptosis.

2.1.7.3 Regulation of MAPK pathway

Mitogen-activated protein kinases (MAPK) are a family of serine/threonine kinases, which connect inflammatory and other extracellular signals to intracellular responses.

MAPK pathway consist of a module of three kinases where MAPKKK activates a MAPKK that activates a MAPK (ERK, JNK, and p38), resulting in the activation of transcription factors like AP-1. Activation of the MAPK pathways may cause the induction of phase II detoxifying enzymes and positively regulate the expression of a number of cytokines genes [44].

Genistein has been found to regulate the molecules in the MAPK pathway in different ways: Genistein is reported to inhibit TGF-β-mediated p38 MAP kinase activation and matrix metalloproteinase type 2 [45]. In other studies, genistein has been found to be effective in preventing cytokine-induced ERK-1/2 activation and promoted apoptotic cell death [46]. Since genistein is a well known inhibitor of tyrosine kinases, it is possible that genistein inhibits tyrosine kinases upstream of p38 and subsequently inhibit the phosphorylation of tyrosine residues on p38, leading to the inactivation of MAPK pathway. On the other hand, genistein also could potentiate the phosphorylation of p38, ERK1/2 in breast cancer lines [47-49].

In summary, the modulation of a large number of signalling pathways is a distinctive property of genistein.

2.2 Phagocytes in the immune system

2.2.1 Immune system

The immune system is a remarkable versatile defence system that has evolved to protect animals from invading pathogenic microorganisms and cancer. It comprises an enormous variety of cells and molecules capable of specifically recognizing and eliminating foreign invaders [50].

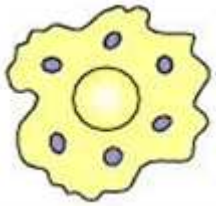
The immune system has two principal components: innate and adaptive immunity, which work in concert to defend the body against infection. The innate immune system recognizes directly a wide variety of microorganisms through highly conserved receptors and essential microbial molecules, such as lipopolysaccharides present on the cell wall of gram-negative bacteria and lipoteichoic acids present in gram-positive bacteria. The cells of the innate immune system represent the first line of defence in the immunosurveillance network. In contrast to the broad reactivity of the innate immune system, the specific components of the adaptive immunity, such as lymphocytes and antibodies, recognize an antigenic challenge to the organism with a high specificity. Once the immune system has

recognized and responded to an antigen, it exhibits immunologic memory; a second encounter with the same antigen induces a faster and more efficient state of immune reactivity. Therefore, life-long immunity against many infectious agents can be acquired after an initial encounter.

2.2.2 Phagocytes and their functions

The most important effector cells of the innate immune system are bone marrow-derived cells that circulate in the blood and migrate into tissues [51]. These include cells of the myeloid lineage including neutrophils, mononuclear phagocytes, and dendritic cells (Tab. 2.3).

Tab. 2.3 Phagocytes and their function in the innate immune system [52]

Cell	Morphology	Activated function
Macrophage		Phagocytosis and activation of bactericidal mechansims
Dendritic cells		Antigen uptake in peripheral sites
Neutrophil		Phagocytosis and activation of bactericidal mechansims

The mononuclear phagocyte system consists of cells that have a common lineage whose primary function is phagocytosis and which play central roles in innate and adaptive immunity. Monocytes, incompletely differentiated after leaving the marrow, are the first cells that enter the blood. Once they enter tissues, these cells mature and differentiate into macrophages. Macrophages are the first phagocytes to encounter invading microorganisms (Fig. 2.2). They are dispersed throughout the body; some take up residence in particular tissues, become fixed macrophages and are named specifically according to different

tissues. For example, in the central nervous system, they are called microglial cells, in the connective tissue histiocytes, in the lung they are called alveolar macrophages and in the liver Kupffer cells. Macrophages rapidly respond to microbes and survive much longer than neutropils at the sites of inflammation. They can undergo cell division at an inflammatory site. Therefore, macrophages are the dominant effector cells of the later stages of the innate immune response, 1 or 2 days after infection [53].

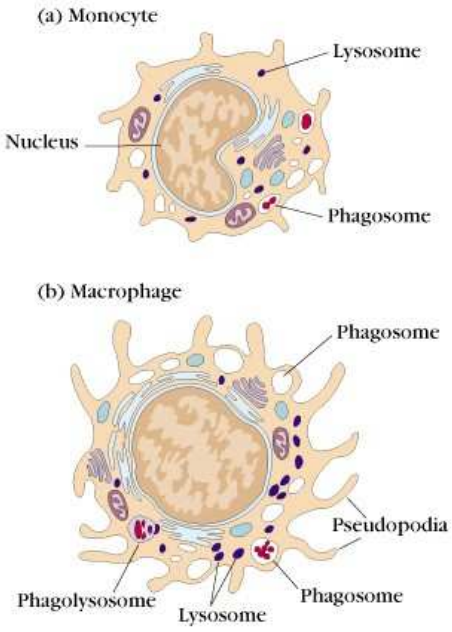


Fig. 2.2 Typical morphology of a monocyte and a macrophage. Macrophages are five to ten fold larger than monocytes and contain more organelles, especially lysosomes [50]

2.2.3 Pattern recognition receptors

Macrophages, neutrophils, and monocytes are the main cells of the host innate immune response that recognize invading pathogens. Monocytes express high levels of Toll-like receptors (TLRs) on their cell membranes, as well as moderate levels of lectin receptors (LRs). During differentiation into macrophages, they retain expression of TLRs while strongly upregulating their expression of LRs [54].

Important differences in the expression of several receptors have also been reported between resident and inflammatory macrophages, as the expression of pattern recognition receptors (PRRs) can be modified by cytokines or microbial products. DCs, which are crucial for antigen processing and presentation, also express most of the PRRs that are important for the recognition of fungal pathogens. Several classes of PRRs such as dectin-1, Toll-like receptors (TLR), families of cytosolic proteins (e.g. NODs, NALPs), and retinoic

acid inducible gene (RIG)-I-like receptors (RLR) have been identified so far [55-57]. These PRRs recognize various pathogen-associated molecular patterns (PAMPs) [58] in a number of cell compartments, such as bacterial lipopolysaccharide or fungal β-glucans, and trigger the release of inflammatory cytokines and type I interferon for host defence [55, 59]. Here we briefly listed the receptors associated with *C. albicans* infections (Fig. 2.3-Fig. 2.4).

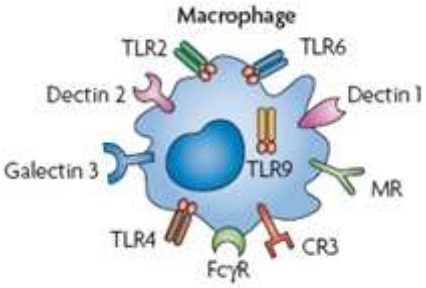


Fig. 2.3 Pattern-recognition receptors on the surface of macrophage involved in *C. albicans* recognition[54]. CR3: complement receptor 3; FcγR: Fcγ receptor; MR: mannose receptor; TLR: Toll-like receptor.

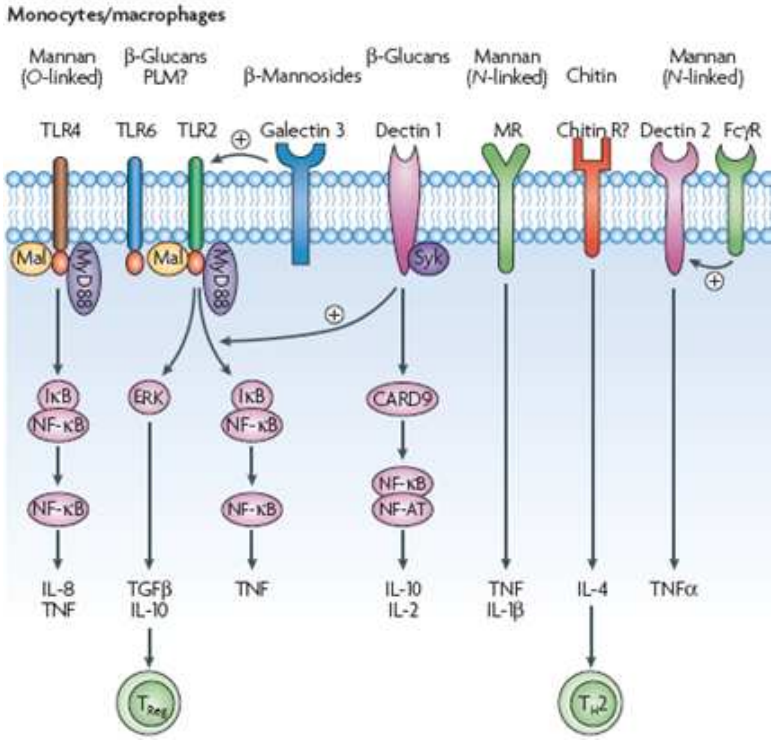


Fig. 2.4 Recognition of *Candida albicans* at the membrane level [54].

2.2.3.1 Toll-like receptors

TLRs recognize various PAMPs derived from viruses, pathogenic bacteria, pathogenic fungi, and parasitic-protozoa. TLRs are expressed in distinct cellular compartments. TLR1,

TLR2, TLR4, TLR5, TLR6, and TLR11 (only expressed in mice) are expressed on the cell surface whereas TLR3, TLR7, TLR8, and TLR9 are expressed in intracellular vesicles such as the endosome and ER. The intracellular TLRs are transported to the intracellular vesicles via UNC93B1, a transmembrane protein, which is localized in the endoplasmatic reticulum of the cell [60-63]. All TLRs differ from each other in ligand specificities, expression patterns, and in the target genes which they can induce [58] (Tab.2.4).

TLRs are type I integral membrane glycoproteins and have a trimodular structure. The extracellular N-terminal domain consists of approximately 16-28 leucine-rich repeats (LRRs) and each LRR consists of 20-30 amino acids with the conserved motif "LxxLxLxxN". The intracellular C-terminal domain is known as the Toll/IL-1 receptor (TIR) domain, which shows homology with that of the IL-1 receptor [58, 59, 64, 65]. This domain is required for the interaction and recruitment of various adaptor molecules to activate the downstream signalling pathway (Fig. 2.5).

Activation of signal transduction pathways downstream of TLRs leads to the induction of the expression of various genes that function in host defence, including inflammatory cytokines, chemokines, major histocompatibility complex (MHC), and co-stimulatory molecules. In this thesis, we were interested in the TLR4 signalling pathways. The TLR4 signalling pathways are categorized into MyD88 (Myeloid differentiation primary response gene 88)-dependent and TRIF (TIR-domain-containing adapter-inducing interferon-β)-dependent pathways [67]. The MyD88-dependent pathway is utilized by all TLRs except TLR3. Stimulation with the TLR ligand recruits MyD88 and the IRAK family of protein kinases to the receptor, which leads to the activation of TRAF6. TRAF6 causes activation of TAK1 via K63-linked polyubiquitination, which results in activation of NF-κB and AP-1 through the IKK complex and MAP kinases, respectively (Fig. 2.5). TLR3 and TLR4 initiate a TRIF-dependent pathway to induce the expression of inflammatory cytokines and type I interferons. The TRIF-dependent pathway activates NF-κB via two independent pathways. The N-terminal domain of TRIF interacts with TRAF6 and activates NF-κB. The c-terminal domain of TRIF interacts with RIP1 and activates TAK1. The TRIF-dependent pathway also induces type I interferon through IRF3. IRF3 is phosphorylated and activated by IKK-related kinases, TBK1, and IKKi (IKKε). TRAF3 acts as a linker between TRIF and TBK1 (Fig. 2.5).

Tab. 2.4 Description of TLRs

TLR	Location of TLR	Ligand	Target pathogen	Signalling adaptor	Transcription factor	Cytokines induced
TLR1/2	Cell surface	Triacylated lipoproteins	Bacterial and mycobacteria	TIRAP,MyD 88	NF-κB	Inflammatory cytokines(TNF-α, IL-6 etc)
TLR2	Cell surface	Peptidoglycan , lipoproteins, zymosan	Gram-positive bacteria *S. aureus*, S. *pneumoniae*, yeast and other fungi	TIRAP,MyD 88	NF-κB	Inflammatory cytokines(TNF-α, IL-6 etc)
TLR3	Endosome	Double stranded RNA (dsRNA)	Viruses	TRIF	NF-κB, IRF3,7	Inflammatory cytokines(TNF-α, IL-6 etc), type I IFNs
TLR4	Cell surface	LPS	Gram-negative bacteria,	MyD88	NF-κB, IRF3,7	Inflammatory cytokines(TNF-α, IL-6 etc), type I IFNs
TLR5	Cell surface	Flagellin	*S. typhimurium*, *P. aeruginosa*	MyD88	NF-κB	Inflammatory cytokines(TNF-α, IL-6 etc),
TLR6/2	Cell surface	Diacylated lipoproteins, zymosan	Mycobacteria, yeast and fungi	TIRAP,MyD 88	NF-κB	Inflammatory cytokines(TNF-α, IL-6 etc),
TLR7/8	Endosome	Single-strande d RNA (ssRNA)	Viruses, HIV, Denge virus influenza	MyD88	NF-κB,IRF 7	Inflammatory cytokines(TNF-α, IL-6 etc), type I IFNs
TLR9	Endosome	CpG DNA	Bacterial DNA	MyD88	NF-κB,IRF 7	Inflammatory cytokines(TNF-α, IL-6 etc), type I IFNs
TLR10	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
TLR11	Cell surface	Profilin, uropathogenic bacteria	*T. gondii* Uropathogenic bacteria	MyD88	NF-κB	Inflammatory cytokines(TNF-α, IL-6 etc),

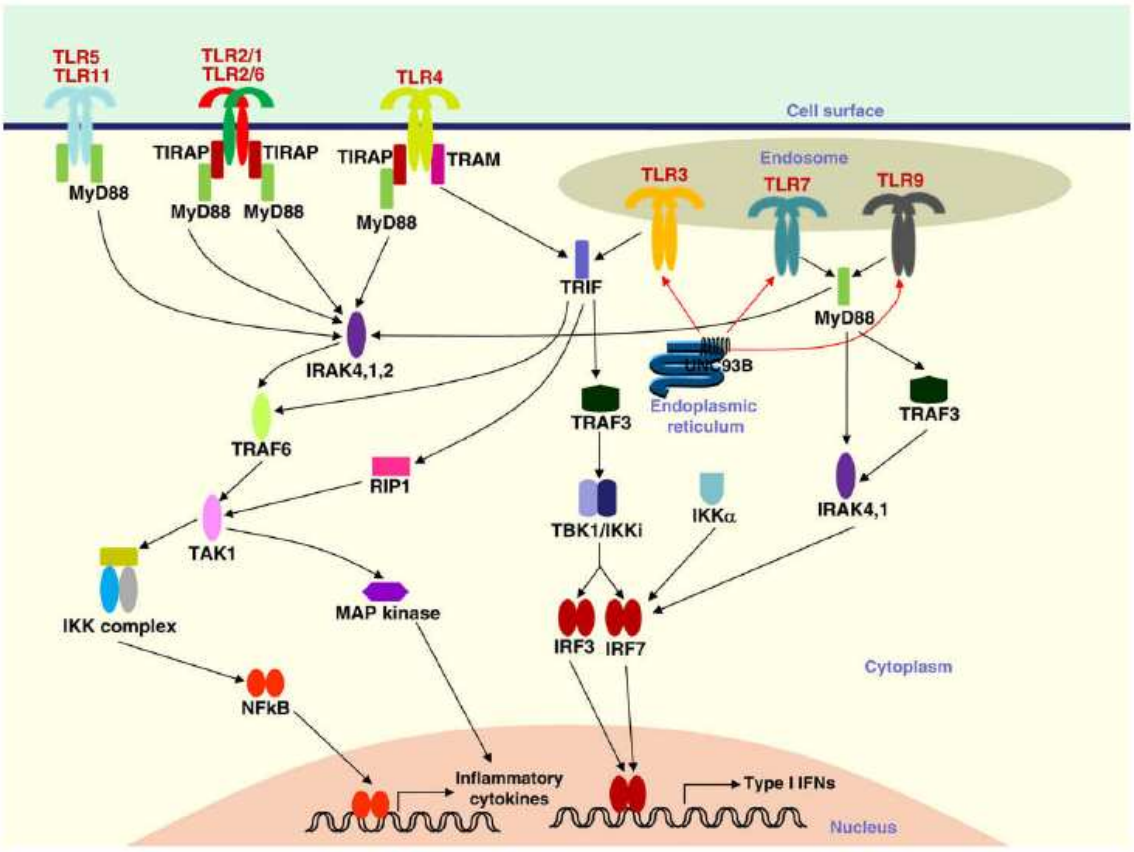


Fig. 2.5 TLR signalling in conventional dendritic cells, macrophages and plasmatoid dendritic cells [66].

2.2.3.2 Dectin-1 receptor

Dectin-1 is a type II transmembrane protein receptor that belongs to the c-type lectin family of receptors, containing an extracellular c-terminal c-type lectin domain, a short stalk region, a single transmembrane domain, and a short 40 amino acid aminoterminal intracellular tail which contains an ITAM (immunoreceptor tyrosine-based activation-like motif) [68]. Dectin-1 can mediate various protective responses through Syk-CARD9 (spleen tyrosine kinases and caspase recruitment domain protein 9)-dependent pathways, such as the stimulation of IL-2, IL-10 [69], and IL−6 [70].

There are two major isoforms of dectin-1, one encoding the full length receptor and a version which lacks the stalk [71]. Murine dectin-1 is widely expressed by many cell types, including macrophages, dendritic cells, monocytes, neutrophils, and a subset of splenic T cells [72]. The expression of human dectin-1 is also found in B cells, eosinophils, and mast cells [73, 74]. Murine dectin-1 expression can be influenced by various cytokines and microbial factors. IL-4, IL-13, and GM-CSF cause a highly up-regulated dectin-1 expression. In contrast, IL-10, LPS, and dexamethasone cause down-regulation of dectin-1

expression [75].

Dectin-1 was initially identified as a dendritic-cell-specific receptor that modulates T cell function through recognition of an unidentified ligand [68, 76]. It was subsequently reidentified as a receptor for β-glucans [77, 78]. Due to its β-glucan specificity, dectin-1 recognizes a number of fungal species, including *C. albicans*, *P. carinii*, *S. cerevisiae*, *Coccidioide sposadasii*, and *Aspergillus fumigatus* [78-83]. The ligation of dectin-1 also triggers intracellular signalling resulting in a variety of cellular responses, including phagocytosis. Apart from Syk-dependent signalling from dectin 1, dectin 1 also collaborates with the TLR2 receptor to stimulate the MAPK (mitogen-activated protein kinase) and NF-κB (nuclear factor-κB) pathways, with subsequent production of pro-inflammatory cytokines such as TNF (tumour necrosis factor) [78, 84].

2.2.3.3 Mannose receptor

The mannan receptor was the first receptor found on the surface of macrophages. It is a c-type lectin mannose receptor (MR) [85, 86], which recognizes oligosaccharides that terminate in mannose, fucose, and N-Acetylglucosamine (GlcNAc) [87]. This binding is mediated by 4 to 8 carbohydrate-recognition domains (CRDs) in the extracellular region of the receptor [88]. MR preferentially recognizes α-linked oligomannoses with branched, rather than linear, structures from *C. albicans* in monocytes and macrophages [89, 90], resulting in important protective effects against *C. albicans* infections such as stimulation of cytokine production, induction of DC maturation, and T cell immunity [54].

2.2.3.4 Interferon gamma receptor

Interferon γ (IFN γ) is released in an immune response by T lymphocytes and natural killer (NK) cells and plays an important role in the host defense against virus and mycobacteria [91]. The receptor for IFN γ is expressed on lymphoid cells, including monocytes, macrophages, T cells, B cells, NK cells, and nonlymphoid cells such as fibroblasts and endothelial cells [92]. IFN γ acts on macrophages to increase ability to resist the growth of intracellular pathogens by enhancing production of antimicrobial effector molecules, such as reactive oxygen species (ROS) and nitric oxide (NO) [93, 94]. The receptor complex consists of the heterodimer of two chains: IFNGR1 and IFNGR2a. Binding of IFN γ to the receptor activates the Jak2-Stat1 signaling pathway [95, 96]. The receptor-associated Jak1 and Jak2 tyrosine kinases phosphorylate the IFNGR-1 chain. The

tyrosine-phosphorylated receptor recruits signal transducer and activator of transcription 1 (Stat1) to the receptor, resulting in the tyrosine phosphorylation of Stat1. The phosphorylated Stat1 forms dimers and translocates to the nucleus and, after binding to a specific sequence in the promoter region of IFN-γ-inducible genes, induces gene transcription.

2.2.4 Specific kinases involved in pathways activated by LPS and IFN-γ

Kinases and phosphatases play an important role in signal transduction casacades activated by LPS and IFN-γ in macrophages (Fig. 2.6). Already in 1991, LPS was reported to induce tyrosine phosphorylation of several proteins, and two of these tyrosine phosphoproteins of 41 and 44 kDa are isoforms of mitogen-activated protein (MAP) kinase [97-99]. Tyrosine kinase Jak2 was also reported to be involved in LPS-induced macrophage activation by regulating the PI3 kinase and the MAP kinase JNK which controls the expression of inflammatory factors affecting the release of IL-1β [100]. Meanwhile, LPS can activate the human monocyte PI3K pathway [101, 102]. The tyrosine kinase Syk plays an important role in the activation cascade in rat alveolar and peritoneal macrophages *in vitro,* and in the human THP-1 cell line [103]. Src tyrosine kinase inhibitor PP1 was found to inhibit LPS-induced macrophage activation, iNOS expression, and TNF-α production [104, 105].

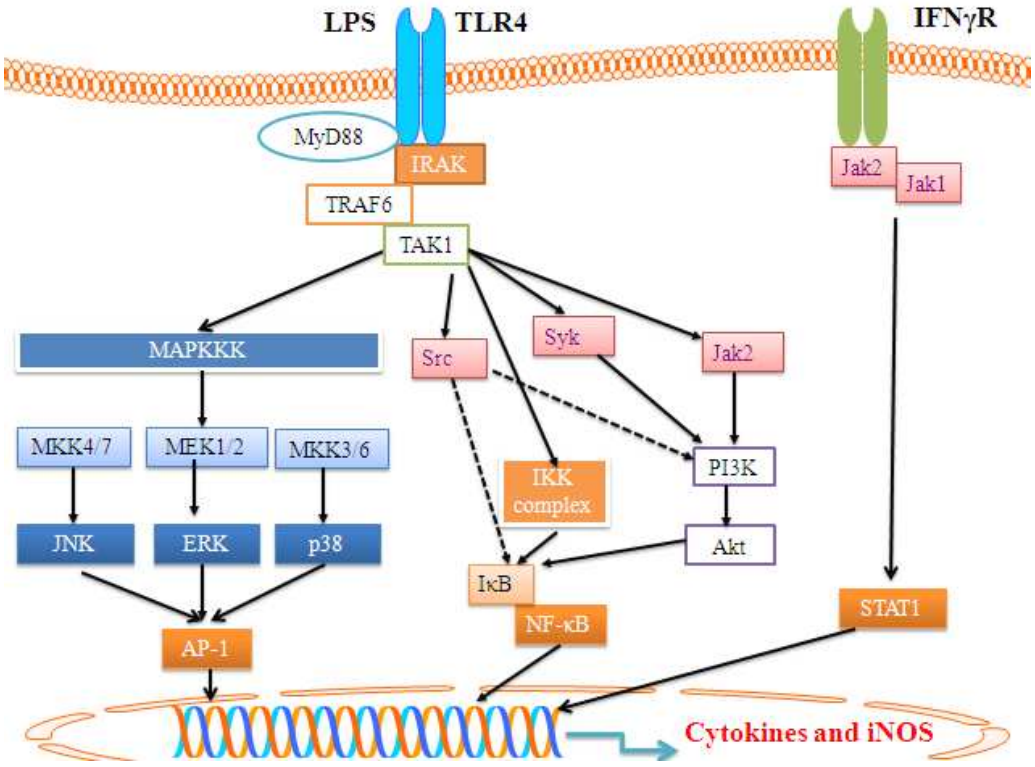


Fig. 2.6 Scheme of kinase involved in the LPS and IFN-γ activated macrophage signaling pathways according to the literature. Black arrows show the known connection, dash arrows mean that the mechanism is not clear yet.

2.2.5 Phagocytosis

Phagocytosis is the process of internalization of particles by cells: in the first step in phagocytosis, macrophages are attracted by and move toward a variety of substances generated in an immune response; this process is called chemotaxis. The next step in phagocytosis is adherence of the antigen to the macrophage cell membrane. Complex antigens, such as whole microbial cells or viral particles, tend to adhere well and are readily phagocytosed; isolated proteins and encapsulated bacteria tend to adhere poorly and are less readily phagocytosed. Adherence induces membrane protrusions in the mammalian cells called pseudopodia, which extend around the attach materials. Fusion of the pseudopodia encloses the material within a membrane-bound structure called phagosome and then enters the endocytic processing pathway. In this pathway, the phagosome moves toward the cell interior, where it fuses with a lysosome to form a phagolysosome. Lysosomes contain lysozyme and a variety of other hydrolytic enzymes that digest the ingested material. The digested contents of the phagolysosome are then eliminated in a process called exocytosis.

The macrophage membrane has receptors for certain classes of antibodies. If an antigen is coated with the appropriate antibody, the complex of antigen and antibody binds to antibody receptors on the macrophage membrane more readily than antigen alone and phagocytosis is enhanced [50].

2.2.6 Killing of phagocytosed microbes

Activated macrophages kill phagocytosed microbes by the action of microbicidal molecules in phagolysosomes: activated macrophages produce several proteolytic enzymes in the phagolysosomes, which function to destroy microbes. Moreover, reactive oxygen species are produced which are highly reactive oxidizing agents that destroy microbes. In addition to reactive oxygen species, macrophages produce reactive nitrogen intermediates, mainly nitric oxide (NO), by the action of an enzyme called inducible nitric oxide synthase (iNOS). However, when macrophages are strongly activated, they can injure normal host tissues by the release of lysosomal enzymes ROS and NO. The microbicidal products of these cells do not distinguish between self tissue and microbes. As a result, if these products enter the extracellular environment, they are capable of causing host tissue injury [50].

2.3 Candida albicans

2.3.1 Background

C. albicans is a polymorphic fungus from the Ascomycota, diverged from *S. cerevisiae* around 200 million years ago [106, 107]. *C. albicans* is able to grow in a variety of morphological forms. These range from unicellular budding yeast (Fig. 2.7 A) to hyphae (Fig. 2.7 C) [106, 108]. In between these two morpholgy, the fungus can form pseudohyphae. Pseudohyphae resemble hyphae, but are morphologically different. Hyphae and pseduhyphae are both important virulence factors. Hyphae formation can be triggered by specific environmental conditions, such as temperature of 37 °C, neutral or alkaline pHs, increased CO_2 concentrations, or the presence of serum [107] (Fig. 2.7). *C. albicans* also expresses several other virulence factors that contribute to pathogenesis. These factors include adhesions, secreted aspartyl proteases, and phospholipases [109].

Fig. 2.7 Yeast, hyphal and pseudohyphal morphologies [107] (A) budding yeast (B) pseudohyphae (C) hyphae

C. albicans is a ubiquitous fungal organism that often colonizes the skin and the mucosal surfaces of healthy individuals without causing disease. However, when the normal host defence mechanisms are impaired (for example, in patients who are undergoing chemotherapy for malignancies, receiving immunosuppressants after an organ transplantion, or patients with AIDS), *C. albicans* can become a pathogen. Candidaemia has an incidence of between 1.1 and 24 cases per 100,000 individuals and an associated mortality rate of more than 30 % [110, 111].

Currently, several classes of systemic antifungal compounds are applied in the clinical practice. They target pathways related to the structure and synthesis of the fungal cell membrane or cell wall. Fluconazole and other azoles lead to the depletion of ergosterol, an essential component of the cell membrane; amphotericin B interacts with sterols in the outer cell membrane, leading to changes in permeability and leakage of cytosolic components; and echinocandins inhibit synthesis of β-glucans of fungal cell walls. The application of these antimycotic agents are challenged by the increasing numbers of resistant strains and severe side effects [112]. Therefore, there are desperate needs for new therapeutics addressing new targets.

2.3.2 The MAP kinases signal transduction network in C. albicans

MAP (mitogen-activated protein) kinases signal transduction pathways are widespread in eukaryotic cells to couple environmental responses to transcriptional regulation. The core structure of MAPK pathway is highly conserved, they comprise three kinases: the MAP kinase (MAPK), the MAP kinase kinase (MAPKK), and the MAP kinase kinase kinase (MAPKKK). When the MAPKKK is activated by upstream signals, it becomes phoshorylated and in turn phosphorylates the MAPKK which, in turn, does so to the MAPK. The MAPK usually transmits the signal to downstream transcription factors that generate a

specific adaptive response. These pathways have been extensively characterized in the non-pathogenic model yeast species *S.cerevisiae* and *Schizosaccharomyces pombe*. In *S. cerevisiae* they are involved in several different processes, such as adaptation to high osmolarity (HOG pathway), mating, growth, and cell integrity (PKC1-mediated pathway) (Fig. 2.8). Proteins homologous to the *S. cerevisiae* MAPK have been identified in *C. albicans* and are designated Cek1p, Hog1p, Mkc1p, Cek2p, and Csk1p, respectively.

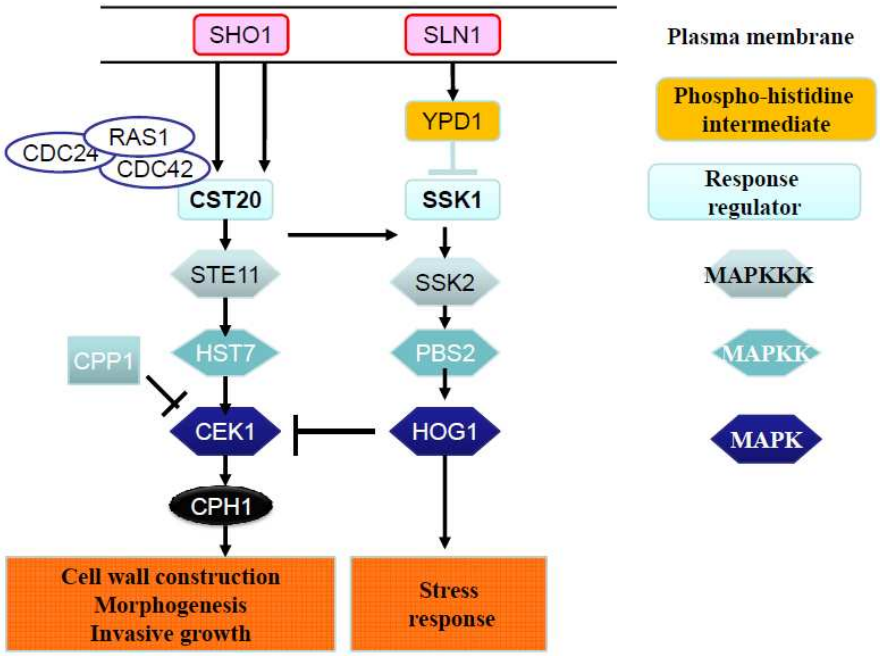


Fig. 2.8 HOG and Cek1 mitogen-activated protein kinases signalling in *C. albicans* [113].

2.3.2.1 The HOG pathway

The HOG pathway in *C. albicans* is implicated in several stress resistance mechanisms (osmotic, oxidative, temperature, and antifungal drugs), such as regulation of cell wall biosynthesis (adherence to host cells), white-opaque switching, morphogenesis, and virulence of the organism [114].

In *C. albicans*, this pathway is activated by the plasma membrane protein CaSln1p, a sensor histidine kinase forming a two-component system with Ypd1p. Another two cytoplasmic histidine kinases, Chk1p [115] and CaNik1p [116], are also involved in osmosensing, morphogenesis, and cell wall biogenesis [114]. Under non-stress conditions, Sln1p phosphorylates itself, which leads to the constitutive activation of CaSln1p and Ypd1p. The phosphate is then transferred to CaSsk1p, which inhibits the activation of Ssk2p and CaSsk22p. Under hyperosmotic stress inducing conditions, the initial step of

phosphorylation in the cascade is inhibited, allowing phosphorylation of Ssk2p and CaSsk22p and hence the activation of the MAPK pathway through Pbs2p and Hog1p. Phosphorylation of Hog1p can activate the transcriptional activators Hot1p, Msn2p, Msn4p, and Skn7p, or the repressor Sko1p [117]. Deletion mutants of *CaHOG1* are sensitive to osmotic stress and resistant to compounds (nikkomycin Z, Congo red, and calcofluor white) that interfere with cell wall function. *CaHOG1* also has a repressive effect on pseudohyphal and filamentous growth. Deletion of *CaHOG1* also results in a drastic decrease in mortality in systemically infected mice, suggesting a role for this MAP kinase pathway for the virulence of *C. albicans* [118].

2.3.2.2 The Cek1-mediated pathway

The Cek1p MAPK pathway was first studied in 1992 by Whiteway et al. and is known in *C. albicans* for its role in growth [119], mating [120], and cell wall construction [121]. This pathway is activated by the small GTPase Cst20p, subsequently acting upon the MAPKKK Ste11p followed by Hst7p, Cek1p, and finishing with the activation of the transcription factor Cph1p (Fig. 2.8). Disruption of *CEK1* causes defects in switching from unicellular budding growth to invasive hyphal growth on certain media, such as Lee's or Spider medium. *CEK1* gene codes for a virulence determinant of *C. albicans* both in systemic murine candidiasis and localized murine candidiasis [119] [122].

2.3.3 Cell wall structure

The fungal cell wall is an essential organelle that maintains the viability of fungal cells. Knowledge of the structure of the cell wall is necessary to understand how it is recognized by the host immune system. Fungal cell walls combine skeletal and matrix components. The skeletal component of the cell wall of *C. albicans* is a core structure of β-(1,3)-glucan covalently linked to β-(1,6)-glucan and chitin (αβ-(1,4)-linked polymer of *N*−acetylglucosamine (GlcNAc))(Fig. 2.9). These polymers form hydrogen bonds between adjacent polysaccharide chains to form a tough three-dimensional network of microfibrils. The skeletal components of the cell wall are always found close to the cell membrane in an inner layer, although some chitin and glucan can be present throughout the whole wall. In budding yeast cells, chitin and β-(1,3)-glucan can become exposed at the surface of budding scar [123]. In addition to the glucan and chitin skeleton, the *C. albicans* cell wall contains a matrix that mainly comprises glycosylated proteins. The major class of cell wall

proteins are glycosylphosphatidylinositol (GPI)-anchor-dependent cell wall proteins (GPI-CWPs), which are attached through a GPI remnant to β-(1,3)-glucan or chitin by a highly branched β-(1,6)-glucan linker. The CWPs are normally highly glycosylated with mannose-containing polysaccharides (sometimes called mannan), and carbohydrates can account for up to 90% of their molecular mass. Many CWPs have a lollipop structure with a globular domain that is presented to the outside of the cell and a Ser/Thr-rich polypeptide stem-like domain that is stabilized in the cell wall by *O*-linked mannan side chains. These ether-linked *O*−mannans are relatively short linear polysaccharides that consist of one to five mannose (Man) sugars, that are almost exclusively α-(1,2)-linked (Fig. 2.9). *N*-mannan consists of a core $Man_8GlcNAc_2$ triantennary complex to which a highly branched structure is attached, comprising up to 150 mannose sugars arranged as an α-(1,6)-linked backbone with side chains of α-(1,2)-, α-(1,3)-mannose and phosphomannan (Fig. 2.9).

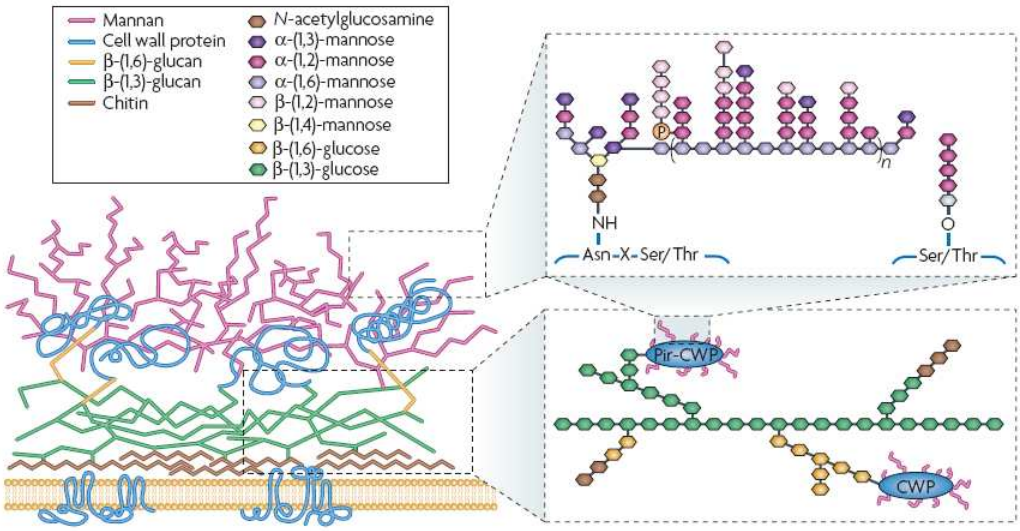


Fig. 2.9 The structure of the *C. albicans* cell wall[54]. Cwp stands for cell wall protein.

2.3.4 Respiratory chain

Mitochondria are known as the power houses of the cell, they play very important roles in a range of processes such as ATP production, electron transport, and oxidative phosphorylation, release of caspase-activating proteins, generation of reactive oxygen species (ROS) and changes of cellular redox potentials [124]. Due to these properties, mitochondria are considered to be a potential target for antifungal agents. Compounds such as rotenone, antimycin, myxothiazols, melithiazols and cystothiazoles are known to exert

their antifungal activity by specifically inhibiting the electron transport within the respiratory chain [125, 126]..

The complex structure of the respiratory chain in *C. albicans* is not yet fully elucidated. As in mammalian cells, the conventional respiratory chain of *C. albicans* comprises four large respiratory chain enzyme complexes (Fig. 2.10): NADH–ubiquinone oxidoreductase (complex I), succinate dehydrogenase (complex II), ubiquinol–cytochrome c (cytochromes bc1, complex III), and cytochrome c oxidase which is the terminal oxidase (cytochrome aa3, complex IV). In each complex, electron transport is coupled to proton translocation, with the resultant proton motive force being used for ATP synthesis. Specific inhibitors are known for each complex: rotenone inhibits complex I, TTFA inhibits complex II, antimycin A and myxothiazol inhibit complex III, and cyanide inhibits complex IV. This conventional respiratory chain provides a conserved route for passaging of electrons from NADH to molecular oxygen.

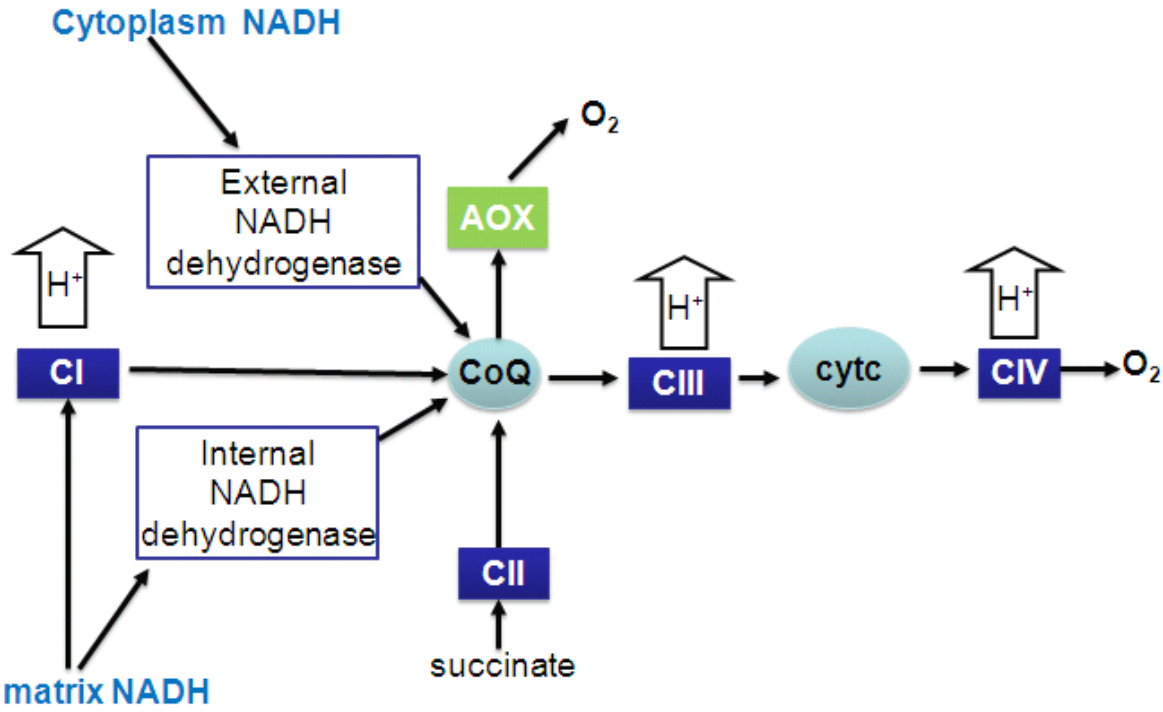


Fig. 2.10 Structure of respiratory chain of *C. albicans* [127]

Additional NADH dehydrogenases (internal and external, facing the mitochondrial matrix and intermembrane space) act in parallel with complex I [128]. These dehydrogenases do not translocate protons [129] and are only partially sensitive to rotenone.

In addition, *C. albicans*, in common with many other fungal species, expresses an alternative oxidase (AOX) that branches off at the level of coenzyme Q (CoQ) in response to changing environmental conditions or cellular energy demands [130, 131]. It is induced by treatment with inhibitors of complex III and IV, such as antimycin A or cyanide [132].

Also in *S. cerevisiae*, elements of the conserved respiratory chain are present, but the complex I is absent [133]. Instead, *S. cerevisiae* has three NADH dehydrogenases associated with the inner mitochondrial membrane capable of coupling the oxidation of NADH to the reduction of CoQ [134-136] (Fig. 2.11). One internal NADH dehydrogenase (NDI) faces the matrix space and utilizes mitochondrial NADH; the other two external NADH dehydrogenases face outward and can receive electrons from cytosolic NADH (NDE_1 and NDE_2).

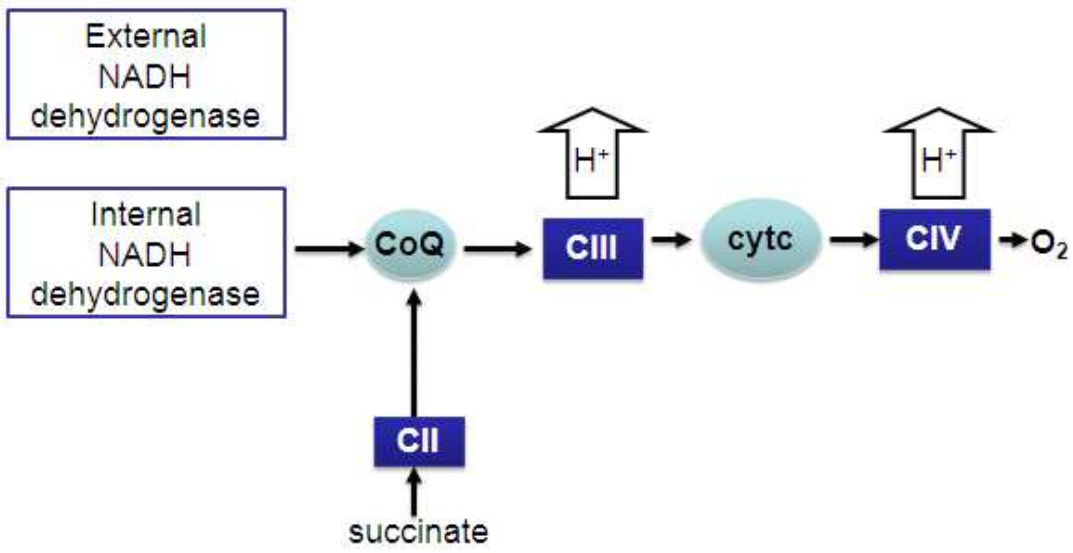


Fig. 2.11 Structure of respiratory chain of *S. cerevisiae*

2.4 Thesis aim and objectives

The aim of the thesis is to investigate the role of genistein in *C. albicans* infections. The activity of genistein was examined using a murine macrophage cell line *in vitro*. The design of the experiments is outlined in Fig. 2.12.

Experiments were designed to investigate the following specific objectives:

- ✧ To identify potentential effects of genistein on the properties of the immune cells including (i) anti-inflammatory effects of genistein and (ii) elucidation of the underlying mechanism
- ✧ To determine the molecular effects of genistein on the pathogen *C. albicans* focusing on the modulation of the function of mitochondria of *C. albicans*
- ✧ To examine the effects of genistein on the interaction of macrophages and *C. albicans*
- ✧ To examine the role of single genes on the interaction of macrophages and *C. albicans* by using deletion mutants and identify the action of genistein on the mutants.

Previous investigations have demonstrated that genistein shows a variety of biological activities. For example, genistein can function as a phytoestrogen, an antioxidant, and an inhibitor of a broad range of tyrosine kinases. Those lead to its chemoprotectant activities against different types of cancers, protection of cardiovascular disease, chronic inflammatory disorders, and antiviral properties. As *C. albicans* is an opportunistic pathogen, the immune situation plays a vital role for the protection against *C. albicans* infections. An impaired immune system increases the risks to acquire opportunistic infections and uncontrolled neoplastic tissue growth; while an over-activated immune system could lead to inflammation, allergy, and autoimmunity. Therefore, we first investigated the effects of genistein on immune cells represented by the macrophages cell line RAW264.7. We tested the effects of genistein on macrophages including effects on cell proliferation, cell morphology, cell cycle, cell skeleton structure, and anti-inflammatory effects against different stimuli from bacteria and cytokines. In order to determine the mechanisms, we utilized a reporter cell line (RAW- Blue^{TM} cells) that was stably transfected to express a secreted embryonic alkaline phosphatase (SEAP) gene inducible by NF-κB and

AP-1 transcription factors. Quantitative real time PCR arrays and western blotting were used to further validate the alteration in gene expression and to investigate the phosphorylation of certain proteins (Fig. 2.12, Section 5.1 and 5.2).

The investigations of the action of genistein on *C. albicans* focused on the mitochondria function. Oxygen uptake and the measurement of reactive oxygen species production were examined and compared with the action of specific respiration chain inhibitor in both organisms *C. albicans* and *S. cerevisiae*. The metabolic activity of *C. albicans* after genistein treatment was also determined by enzymatic assay (Fig. 2.12, section 5.3).

The effects of genistein against *C. albicans* infection were determined in two ways. On one hand, genistein-pretreated macrophages were infected with *C. albicans*. On the other hand, macrophages were infected with *C. albicans* which were treated with genistein. Phagocytic efficiency and cytokine production were detected. The mechanisms were investigated in both infective conditions. Cell wall components analysis and cell surface hydrophobicity were further detected to elucidate the mechanisms.

The MAP kinase pathways are related to cell wall integrity and regarded as important virulence factors regulator for *C. albicans*. We investigated the influence of single MAP kinase pathway gene deletions of *C. albicans* on the cell wall components structure and the phagocytic efficiency by macrophages. We further investigated the genistein effects on phagocytosis of the mutants to further elucidate the activity of genistein against *C. albicans* infection (Fig. 2.12, section 5.4).

2.5 Experimental design

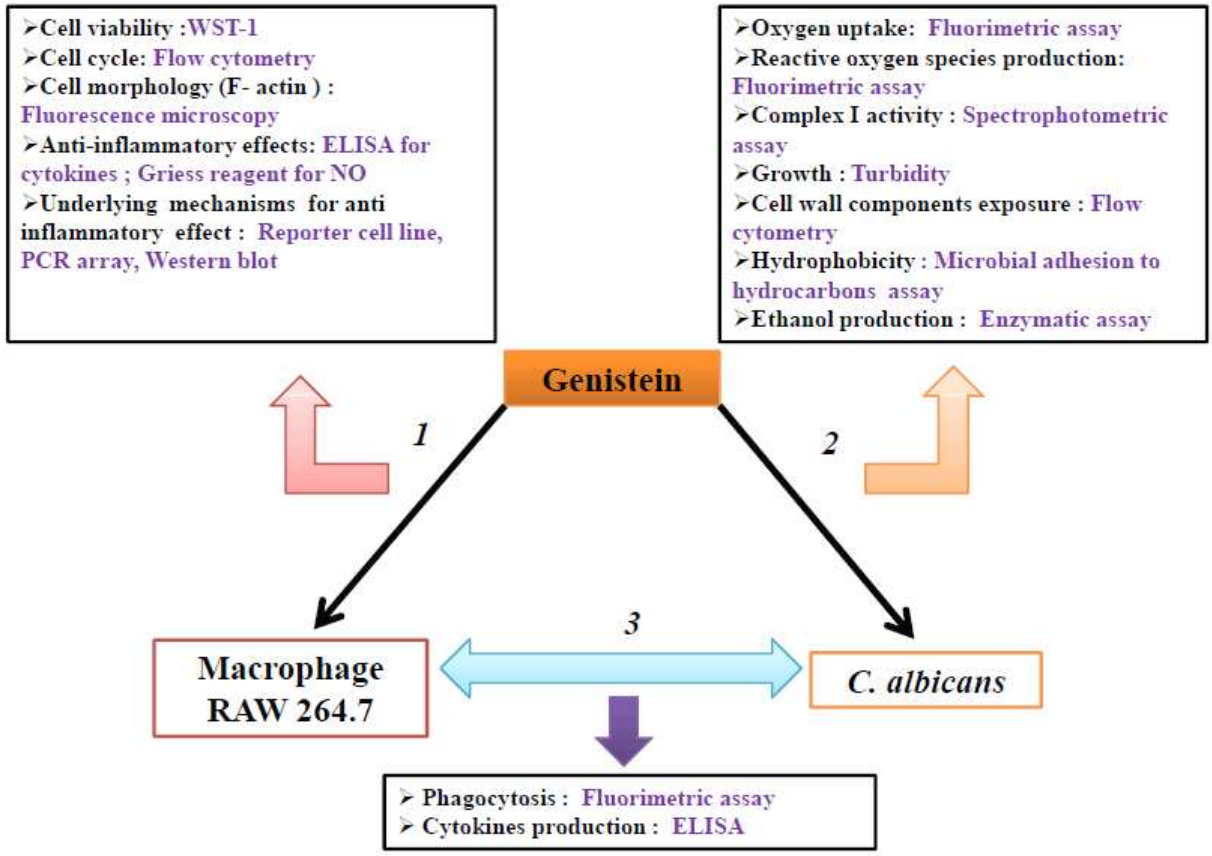


Fig. 2.12 Flowchart outlining overall design of experiments undertaken in the thesis: Words in purple color represent the methods used. Part 1 shows experiments designed for investigation the influence of pre-treated macrophage with genistein. Part 2 points out experiments designed for investigation of the influence of pre-treated *C. albicans* with genistein. Part 3 shows the interaction between genistein-treated macrophage with *C. albicans* as well as the interaction between genistein-treated *C. albicans* with macrophages

3 MATERIALS AND METHODS

3.1 Equipment and materials

Analyse balance	PC4400, Mettler Sartorius
CO_2 Incubator	CO_2-Auto-Zero, Heraeus
Bunsen burner	Fireboy, Heraeus
Steam sterilizer	464, Sauter GTA40, Biomedis
Flow cytometry & Software	FACS Canto & FACS Diva 5.0, BD Bioscience
Flow cytometry & Software	FACS Canto & FACS Diva 5.0, BD Bioscience
Fluorescence microscope	Kenyence
Culture shaker	Pilot-Shake, Kühner
Magnet mixer	MR 2002, Heidolph
Multi-well-Fluorimeter	CytoFluor® Series 4000, PerSeptive Biosystems Synergy 4® BioTek
pH meter	pH 211 Microprocessor pH Meter, Hanna Instruments
Pipettes	Research 0.1-2.5 µl 1520615, Research 2-20 µl 3478105, Research 20-200 µl 3612935, Research 100-1000 µl 3633985, Research Multichannel 10-100 µl 3263575, Research Multichannel 30-300 µl 3582685, Eppendorf Transferpette® -8 10-100 µl 01F72011, Transferpette® -12 15-300 µl 03D5931, Brand
Pipettinghelper	Easypet® 4412, Eppendorf CellMate II, Matrix
Plateshaker	Titramax 1000, Heidolph
Shaker	Heidolph Innova 2100, New Brunswick

	Scientific
Microtiter plate reader & Software	µQuant & KCjunior Installation, BioTek Instruments
Sterile bench	HLB 2448 & HBB2472 S, Heraeus Hera Safe, Therma Electron Corp.
Thermomixer	5437, Eppendorf
Oven	Memmert
Vortexer	Vortex Genie 2, Scientific Industries VF2, IKA®-Labortechnik
Water system	MilliQ Water Purification Systems, Millipore
Water bath	Köttermann
Hemocytometer	*Neubauer improved*, Assistent Germany
Centrifuge	5804R & 5402, Eppendorf Sepatech Megafuge1.0 & Biofuge *fresco*, Heraeus
ELISA washer	BioTek
Needles	TSK-SUPRA 1.1x100, Luer
Combitips	10 ml 1=0.2 ml 12307 & 500 µl 1=10 µl 360407, Eppendorf
Single-use pipette	Costar Stipette 5 ml 4487, 10 ml 4488 & 25 ml 4489, Corning
Cryovials	Nalgene® Cryoware™ 5000-0020, Nalge Nunc Int.
Multi-well-culture plate	4 Well Nunclon Surface 176740, Nunc 96 Well Tissue Culture Plate Flat Bottom 353916, BD Falcon
Pipette Tips	epT.I.P.S. Standard 2-200 µl 0030000.870, epT.I.P.S. Standard 50-1000 µl 0030000.919, Eppendorf

Reaction tube	Safe-Lock Tubes 1.5ml 0030120.86, Eppendorf
Resevior	60 ml solution volume with cover, Eppendorf
Cell culture flask	Corning® Flask 0.2 µM Vent Cap Angled Neck: 175 cm^2 431080 & 75 cm^2 430641, Corning Tissue Culture Flask 25 cm^2 PS red filter cap 690175, Greiner Bio-one
Cell scraper	23 cm 179693 & 32 cm 179707, Nalge Nunc
Centrifuge tubes	15 ml volume AN77.1 Roth 50 ml volume 430291 & 430921, Corning
Oxoplate®	Presens, Regensburg

3.2 Methods

3.2.1 Sterile Conditions

All work with yeasts and phagocytic cells were conducted under sterile conditions. Glassware (shaker flask, glass pipettes, etc.) were sterilized at 180 °C for six hours. Solutions, media and plastic materials (eg. pipette tips) were steam sterilized at 121 °C for 20 min; alternatively, solutions that were not suitable for steam sterilization were sterilized with a filter pore size of 0.2 microns (Millipore). All ready to use media and materials were purchased sterile from manufacturer.

3.2.2 Preparing PBS

The buffered, isotonic saline PBS (phosphate buffered saline) from tablets of Invitrogen (18912-014), was prepared according to manufacturers instructions: one pill (5 g) is solved in 500 ml MilliQ-water, and adjusted to pH=7.4 with hydrochloric acid (HCl).

3.2.3 Cell number counting

The determination of the number of yeast and macrophages cells was carried out by using a Neubauer improved Hemocytometer (Fig. 3.1). It consists of nine large squares, each with an area of 1 mm^2. Largest square in the center is divided into 5 × 5 small squares (each 0.04 mm^2), which in turn consists of 4 × 4 midget squares (each 0.0025 mm^2). The chamber depth is 0.1 mm. For the cell counting, the chamber was filled with 10 µl well mixed cell suspension and the cells were

counted under the microscope. The macrophages were counted within the four corner squares (big red square); cells which were located on the outer boundary lines were not been included. Subsequently, cell number was divided by four in order to obtain the mean value for each corner squares. The volume per corner square of a chamber is 0.1 µl, so this average was multiplied by 1×10^4 in order to determine the number of cells per milliliter. For the determination of the concentration of yeast, cells were counted for five small squares (small red square) within the central large squares. The amount corresponded to the number of cells per 0.02 µl cell suspension, so this number was multiplied by 5×10^4 to identify the yeast concentration per milliliter. To avoid too high cell density, the counting was carried out by dilution of the cell suspensions in PBS and the dilution factor was included in the calculation of cell number.

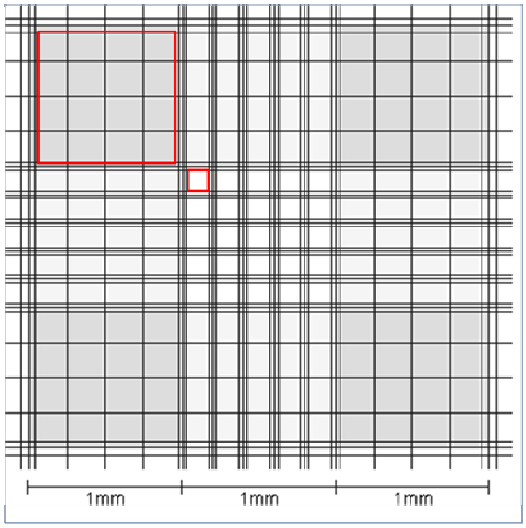


Fig. 3.1 Scheme of Neubauer improved Hemocytometer [137]

3.2.4 Data analysis and Programmes

The text, graphics and tables of this work were created with the programes Microsoft® office 2007 (Microsoft Corp.) and OriginPro70 (Origin Lab Corp.). Significance of the data was evaluated using the student's t-test.

3.3 Cell culture

3.3.1 Cell culture media and ingredients

In Tab. 3.1，the cell culture medium and ingredients are listed. The DME medium was stored at 4 °C. 5 ml Pen-Strep solution and 50 ml FBS were added to 500 ml of DME medium before use. For RAW-Blue™ cells, 200 µg/ml Zeocin™ was additionally added to the prepared DMEM,

FBS was previously heat treated at 55 °C for 30 min. The media were warmed in a water bath at 37 °C before use. The freezing medium was purchased and stored in 5 ml aliquots at -20 °C.

Tab. 3.1 Cell culture medium and components

	Company	Article number
DMEM *(Dulbecco's modification of Eagle's medium)*	Lonza	BE12-741F
Frozen medium (with DMSO)	Invitrogen	12648-010
FBS (*fetal bovine serum)*	Lonza	14-801F
100x Pen-Strep *(Penicillin-Streptomycin)*	Sigma	P0781
Zeocin™	Invivogen	ant-zn-5b

3.3.2 RAW264.7

RAW264.7 cells are murine macrophages [138]. The cell line was purchased from the American Type Culture Collection (ATCC; TIB-71) and was cultivated immediately as indicated by ATCC after delivery.

3.3.3 RAW-Blue™

RAW-Blue™ cells (InvivoGen; #raw-sp), derived from murine macrophages, are designed for the study of pattern recognition receptors (PRRs). They express a large repertoire of different classes of PRRs, such as the Toll-like receptors (TLRs), RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs).

They stably express a secreted embryonic alkaline phosphatase (SEAP) gene inducible by NF-κB and AP-1 transcription factors. RAW-Blue™ cells are resistant to the selection marker Zeocin™. Upon TLR, RLR or NOD stimulation, RAW-Blue™ cells activate NF-κB and/or AP-1 leading to the secretion of SEAP which is easily detectable and measurable when using QUANTI-Blue™ as substrate.

3.3.4 Cultivation

The adherent RAW264.7 cells were grown in tissue culture flasks at 37 °C and 10 % CO_2. The volume of DMEM was different by the size of the culture flask (Tab. 3.2). After reaching a confluence of approximately 80 %, the cells were harvested and passaged by scraping with a cell scraper and mixing the cells by repeated pipetting up and down with a glass pipette. Finally, an

aliquot (5-10 %) of the culture volume was transferred into a new culture flask and again grown to confluence.

The cultivation of RAW-Blue™ cells is done in the same way as RAW264.7 cells.

Tab. 3.2 Culture volume of different tissue culture flasks

Surface of the bottle [cm^2]	Volume [ml]
25	10
75	30
175	50

3.3.5 Long-term storage

80 % of confluent cells in a 25 cm^2 culture flask were washed with 10 ml PBS, scraped, transferred into a 15 ml centrifuge tube and harvested by centrifugation (3000 rpm, 5 min, 4 °C). Subsequently, the supernatant was decanted; the cell pellet was resuspended in 1 ml of ice cold freezing medium and transferred into cryogenic vials. The cells were stored for 24 hours at -80 °C before they are permanently stored in liquid nitrogen at -196 °C. To reactivate, the cells were thawed as quickly as possible at 37 °C, and transferred into a 25 cm^2 culture flask with 10 ml DMEM medium to dilute the DMSO in the freezing medium. Once the cells had attached the bottom, the medium was changed to the complete removal of DMSO and the cells were cultured as described in (3.3.2).

3.3.6 WST-1 toxicity assay

The determination of cell vitality after a compound treatment was conducted using a tetrazolium salt, which is a redox indicator. In metabolically active cells, the tetrazolium ion is reduced by the dehydrogenases of the respiratory chain to formazan (Fig. 3.2). The formation of formazan results in a color change and can be detected and quantified by measuring the absorbance at the respective wavelength. In our work, the water soluble tetrasolium salt WST-1 (4-[3-(4-Jodophenyl)-2-(4-nitrophenyl)-2H-5-Tetrazolio]-1.3-Benzoldisulfonate) from Roche Diagnostics (11644807001), was used. The reduction of WST-1 results in a water-soluble yellow product, which can be quantified at the wavelength of 450 nm.

100 µl of RAW264.7 cells were seeded into 96-well cell culture plates at 2×10^6 cells / mL, 1×10^6 cells / ml and 5×10^5 cells / ml in DMEM containing various concentrations of genistein

(Sigma: G6649). The cells were incubated for 2 h, 24 h and 48 h under normal cell culture condition. Subsequently, 10 µl WST-1 per well were added and the plate was placed on a plate shaker for 1 min to ensure optimal mixing. After 30 min incubation at 37 °C and 10 % CO_2, the plate was analysed in a microtiter plate reader. For data analysis the mean value and standard deviation were determined from 8 replicates.

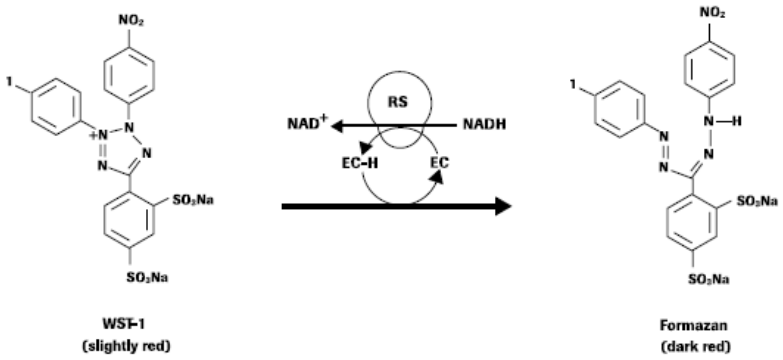


Fig. 3.2: Principle of the toxicity tests cleavage of the tetrazolium salt WST-1 to formazan. (EC = electron coupling reagent RS = mitochondrial succinate-tetrazolium-reductase system) (Invitrogen)

3.3.7 Cell cycle analyses

The distribution of cells at the different stages in the cell cycle was estimated by flow cytometric DNA analysis. Propidium iodide is a fluorescence dye which can bind directly to the DNA in the nucleus, hence the measurement of the fluorescence provides a measure of the amount of dye taken up by the cell and indirectly the amount of DNA content. Cells were harvested and counted when the cells are up to approximately 80 % of confluence, 5×10^5 cells / ml were incubated for 24 h and 48 h with or without various concentrations of genistein. Cells from the different conditions were harvested and washed in the cold phosphate-buffered saline (PBS) pH=7.4, fixed in 70 % cold ethanol and stored at 4 °C until processing. The fixed cells were washed with cold PBS twice, then treated with flurochrome DNA staining solution (0.5 ml) containing propidium iodide (Sigma: P4170) (20 µg / ml) and RNase A (1 mg / ml) (Carl-Roth: 2326466) for 1 hour, than the stained cells were analyzed by flow cytometry. The percentage of cells in each cell cycle phase was calculated by using Flowjo 7.5 software, 10000 cells per sample were be evaluated in each case.

3.3.8 Fluorescence labelling of F-actin and cell nuclei

The RAW264.7 cells were cultured and harvested and the concentration of cells was determined by counting. Subsequently, RAW264.7 cells suspension was prepared with 1×10^5 ml

in DMEM with 2 nM cytochalasin B (Sigma: C6762), 0.1 % DMSO (Biozol: EMR385250) and genistein 25 and 12.5 µM. 500 µl of the respective suspension were seeded into 4 well plates and cultured for 48 hours under standard conditions. Then, the supernatant was removed and the cells were washed twice with 750 µl PBS. After washing, the cells were fixed by addition of 750 µl 3.7 % paraformaldehyde (PFA) for 15 min at room temperature. They were then again washed twice with PBS to remove the PFA and the cells were incubated with 750 µl 0.1 % Triton X in PBS for 5 min to permeabilize. After permeabilization, cells were washed twice with PBS and finally, 500 µl Alexa Fluor® 488-phalloidin (2 U/ml in PBS containing 10% FBS, Invitrogen: 12379) was added for 1 hour at 37 °C in 10 % CO_2. Since phalloidin specifically binds the F-actin, the actin filaments of the cells were fluorescently labeled. Then the cells were washed twice with PBS to remove unbound dye and stored in 500 µl PBS. 1µg/ml of DAPI (Invitrogen: P36931) was added into the wells, It can bind to the nuclei of the cells. Specimens were examined using fluorescence microscope.

3.3.9 Detection of nitric oxide by Griess reagent

Nitric oxide (NO) is an important physiological messenger and effector molecule in many biological systems, including immunological, neuronal and cardiovascular tissues(this depends more to the introduction) The indirect evidence of the release of nitric oxide (NO) by RAW264.7 cells was carried out by Griess reaction [139]. NO, the unstable radical in aqueous solution, reacts with oxygen to form nitrite (NO_2^-) and a smaller amount of nitrate (NO_3^-) [140]. The detection is based on the chemical reaction (Griess-reaction) shown in Fig. 3.3, which uses sulfanilamide and N-1-napthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. The product of this reaction can be detected at 540 nm using a photometer.

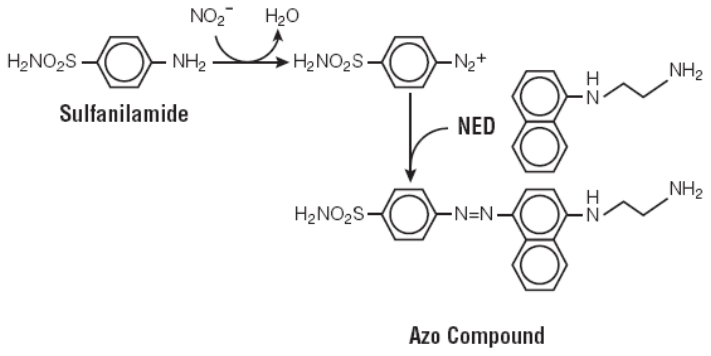


Fig. 3.3 Chemical reactions involved in the measurement of NO_2^- using the Griess Reagent (Roche).

3.3.9.1 Sample preparation

RAW264.7 cells were grown up to a confluence of approximately 80 % in standard conditions of cultivation and counted. 100 µl of 1 × 10^6 / ml cells suspension in DMEM were seeded into the wells of a 96-well plate, so that there were 1×10^5 cells per well. The cells were pretreated with different concentrations of genistein (0-100 µM) or other inhibitors (Tab. 3.3) for 1 hour and then stimulated with LPS (100 ng / ml) for another 20 hours. Finally, 50 µl supernatant per well were transferred by a pipette to a reduced volume 96 well microtiter plate and then used for all other experiments. Cell viability was detected by WST-1 in parellel following the protocol described in 3.3.6.

Tab. 3.3 Specific inhibitors used in the experiment

Inhibitor	**Target**	**Company**	**Solvent**	**Stock concentration**	**Working concentration**
AG490	Jak2 (PTK)	Biaffin(PKI-AG490-010)	DMSO	34 mM	0.16-340 µM
PP2	Src (PTK)	Biaffin(PKI-PP2-001)	DMSO	3.3 mM	0.4-33.3 µM
Syk inhibitor	Syk (PTK)	Calbiochem (574712)	DMSO	14 mM	1.6-14 µM
PD98059	MEK1/2	Biaffin (PKI-PD98059)	DMSO	18 mM	6.7-180 µM
SB203580	P38MAPK	Biaffin (PKI-SB203-001)	DMSO	4 mM	2.2-20 µM
U1026	MEK1/2	Cell signalling (9903)	DMSO	10 mM	10 µM
LY294002×HCL	PI3-Kinase	Sigma (L9908)	DMSO	2 mM	0.27-22.2 µM
Wortmanin	PI3-Kinase	Sigma (W1628)	DMSO	233 µM	0.013-23.3 µM
Akt1/2 inhibitor	Akt1/2	Sigma (A6730)	DMSO	9 mM	0.4-33.4 µM
Bpv(phen)	Phosphatase	AlEXIS Biochemicals(ALX-270-204)	Water	14 mM	1.6-14 µM

3.3.9.2 Detection of nitrite in cell culture

A standard method for detection of sodium nitrite ($NaNO_2$) was used. Griess reagent was prepared by 1:1 mixing sulfanilamide (1 % in 5 % H_3PO_4) and N-(1 naphthyl) ethylendiamid (0.1 % dissolved in 5 % H_3PO_4). Samples prepared in 3.3.9.1 were mixed with 50 µl of the Griess reagent for 10min at room temperature. The calibration was made by dilution of $NaNO_2$ (100 mM $NaNO_2$ in H_2O) to a concentration range from 0 to 100 µM by DMEM, 50 µl of this solution were mixed with 50 µl of the reagent, the samples were measured at 540 nm. For data analysis, a linear regression equation was calculated according to the values of the standard series. With this linear regression equation the nitrite concentration in the cell culture suspension was determined.

3.3.10 Cytokine determination

3.3.10.1 Principle of the ELISA method

The cytokine production by LPS stimulated macrophages was detected by Enzyme linked immunosorbent assay (ELISA) [141]. The principle of the method is shown below:

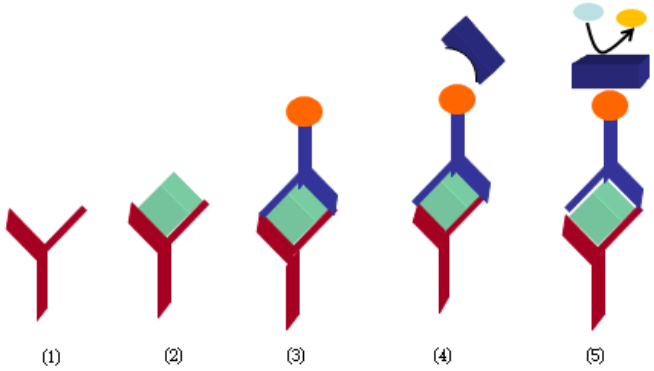


Fig. 3.4 Principle of a sandwich ELISA (1) The ELISA plate is coated with a capture antibody (red); (2) Sample is added, and the respective antigen present binds to capture antibody (green); (3) Biotin-conjugated secondary detection antibody (blue/orange) is added, and binds to the antigen captured by the first antibody; (4) Streptavidin-HRP is added and binds to the biotin conjugated detection antibody (dark blue); (5) Coloured product (light blue) is formed in proportion to the amount of antigen present in the sample; The reaction is terminated by addition of acid and absorbance is measured at 450 nm.

3.3.10.2 Sample preparation

RAW264.7 cells were grown up to a confluence of approximately 80% in standard conditions of cultivation and counted. For TNF-α, 100 µl of 1×10^5 / ml cells suspended in DMEM were seeded into the wells of a 96-well plate. For IL-6 and IL-10, 100 µl of 5×10^5 / ml cells suspended in DMEM were seeded into the plate. The cells were pretreated with different concentrations of genistein or other inhibitors (Tab. 3.3) for 1 hour and then stimulated with 10 µl (1 µg / ml) LPS. The incubation time differed according to the interesting cytokines. For TNF-α determination the concentrations were detected after a incubation time of 1 and a half hour, IL-6 were determined after 5 hours and IL-10 concentrations were determined after 6 hours. Finally, the supernatant was collected and stored at -20 °C. Cell viability was detected by WST-1 in parellel following the protocol described in 3.3.6.

3.3.10.3 ELISA-protocol

Tab. 3.4 Reagents from ELISA READY SET GO! Kit-systems

Coating buffer	PBS-powder dissolved in 1L MillQ H_2O; 00-0044-59
Coating antibody	250 times concentrated, diluted in coating buffer IL-10-Coating: anti-Mouse-IL-10; 88-7104-CP IL-6-Coating: anti-Mouse-IL-6; 88-7064-CP TNFα-Coating: anti-Mouse-TNFα; 88-7324-CP
Block buffer	5 times concentrated in MillQ. H_2O; 00-4202-AD2
Detections antibody	250 times concentrated, diluted in block buffer, biotin-coupled IL-10-Detection: anti-Mouse-IL-10; 88-7104-DT IL-6-Detection: anti-Mouse-IL-6; 88-7064-DT TNFα-Detection: anti-Mouse-TNFα; 88-7324-DT
Avidin-HRP	250 times concentrated, diluted in block buffer; 00-4100-EN2
Substrate	TMB solution, ready to use; 00-4201-SB
Standard	Recombinant mouse cytokine 1µg/ ml, dilution series in DMEM IL-10; 88-7104-ST IL-6; 88-7064-ST TNFα; 88-7324-ST

The reagents used in the assay are listed in Tab. 3.4, the protocol is described as below:

A reduced volume 96 well plate was filled with capture antibody in coating buffer (50 µl / well) and incubated over night at 4 °C. On the next day, the antibody solution was removed and the plate was washed with 150 µl wash buffer (PBS with 0.05 % Tween 20) at least 5 times. To increase the effectiveness of the washes, the plate was blotted on absorbent paper to remove any residual buffer. Then the wells were blocked with 1X assay diluents, incubated at room temperature for 1 hour, washed and 50 µl / well of samples were added. Standard serial dilutions were added to the appropriate wells for 2 hours, after washing, 50 µl / well of detection antibody diluted in 1X assay diluents were added for 1 hour. The plate was washed and then 50 µl / well of AvidinHRP* for 30 minutes were added. This was repeated for a total of 7 washes; then 100 µl / well of substrate solution to each well were added for 15 minutes. Finally, 50 µl of 2 M sulfuric acid (H_2SO_4) were added to each well to stop the enzymatic reaction. The plate was measured plate at 450 nm.

For data analysis, a linear regression equation was calculated according to the values of the standard series. The cytokine concentration in the cell culture suspension was calculated by the linear regression equation. Data were represented as means ±the standard deviation.

3.3.11 Detection of NF-κB and AP-1 activation of RAW-Blue™ Cells

The RAW-Blue™ cells were harvested and counted when the cells were grown up to a confluence of approximately 80 % in standard conditions of cultivation. 180 µl of 5×10^5/ ml of RAW-Blue™ cells were seeded in 96 well plates and treated with genistein (0-100 µM) 1hour before stimulation with 10 µl of LPS (1 µg / ml), incubated in normal culture condition for 20 h. 40 µl of induced RAW-Blue™ cells supernatant were taken into a flat bottom 96 well plate, 160 µL of resuspended QUANT-Blue™ (#rep-qbl) was added, SEAP levels were determined at OD 620nm.

3.3.12 RT² Profiler™ PCR Array (PAMM-018G) for Toll-like receptor pathway

RAW264.7 macrophages were grown up to a confluence of approximately 80 % in standard conditions of cultivation and counted. The cells were seeded in 25 cm^2 flask with the 2×10^5 / ml and cultivated overnight. On the next day, the cells were incubated with or without genistein 50 µM for 1 hour before stimulation with LPS (final concentration 100 ng / ml) for 6 h. Total RNA was prepared from cells using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA was reconstituted in RNase free water; concentration and purity were determined using UV spectrophotometry. RNA integrity was verified by gel electrophoresis.

A total of 0.5 µg RNA was used with RT² First Strand kit (C-03) (Superarray, Fredrick, MD) with the addition of 2 µl of 2GE (5x gDNA elimination buffer). The RNA was incubated at 42°C for 5 minutes and placed on ice of 1 minute. RT cocktail of 4 µl of BC3 (5x RT buffer 3), 1 µl of P2 (primer & external control mix), 2 µl RE3 (RT enzyme mix) and 3 µl of RNase free water was added to each sample. The mixture was carefully pipetted and incubated at 42°C for 15 minutes and then heated at 95 °C for 5 minutes and placed on ice. 91 ml of double distilled water was added to each 20 µl of cDNA synthesis reaction. PCR was performed using 10 µl of the following mixture: 2000 µl of 2x SuperArray RT² qPCR Master Mix, 102 µl diluted first strand cDNA synthesis reaction and 1898 µl of double distilled water in the wells of a 384-well microtiter plate. The amplification process included 1 cycle for 10 mins at 95°C, 40 cycles for 15 secs at 95°C, followed by 40 cycles for 1 min at 60°C.

Thermal cycling and fluorescence detection were performed using LightCycler 480 (Roche). The signals of the target cDNAs were normalized by comparison with the housekeeping genes (GAPDH, Actin, GUSB, HPRT1, HSP90, reverse transcription control, and positive PCR control) supplied within the 384-well microtiter plate. Final calculations were made using a web-based PCR Array Data Analysis tool (Superarray, Fredrick, MD).

3.3.13 Western Blot

RAW264.7 macrophage cells were grown up to a confluence of approximately 80 % in standard conditions of cultivation and counted. The cells were pretreated with or without DMSO (as control), 100 µM genistein, 100 µM PD98059, 12 µM SB203580 or 10 µM U1026 for 1 hour and then stimulated with LPS (100 ng / ml) in the presence of the inbitiors for 15 and 30 min. Cells were washed with ice-cold PBS and scrapped. The whole cell lysates were prepared in ice-cold lysis buffer (50 mM Tris/HCl pH 7.5; 150 mM NaCl; 2 mM EDTA; 1 % Triton; 0.5 % Nonidet p-40 (Sigma); 100 × phosphatase and protease inhibitor cocktail set I (Roche, Calbiochem)). Lysates were clarified by centrifugation. Proteins were quantified by BCA assay and 30 µg protein/lane resolved by electrophoresis on 10-12 % SDS-PAGE. Resolved proteins were transferred onto PVDF membrane (Biorad, USA) by semi-dry transfer in blot buffer (Tris 50 mM, Glycine 40 mM, 0.01 % SDS, and 4 % methanol) at 250 *mA* for 1 hour using the Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Biorad, USA). Membranes were probed with primary antibody followed by HRP conjugated secondary antibody and detection by ECL chemi-illuminescence (RPN2132, Amersham, GE healthcare, USA). All primary antibodies were purchased from cell signal. Phospho-ERK1/2 pathway sample kit (9911), phospho-p38 MAPK (Thr180/Tyr182) (3D7) (9215) and P38 MAP kinase alpha (L53F8) mouse mAb (9228) were used at concentrations of 1:1000. Secondary antibodies were used at dilutions of 1:6666.

3.3.14 Receptor staining of macrophages RAW264.7

RAW264.7 macrophage cells were grown up to a confluence of approximately 80% in standard conditions of cultivation and counted. 1 ml of 10^6 cells / ml were washed with PBS containing 2 % FBS. The surface dectin-1 or TLR2 receptor expressions were detected respectively using TLR2 mAb (SC-52736, Santa Cruz Biotechnology, USA) or recombinant mouse Dectin-1/CLECA7 mAb (1756-DC-050, R&D Systems, USA). The cells were incubated for 1 hour with 200 µl of the antibody solution which was prepared by a 1 / 50 dilution of the delivered antibody. For detection of the primary antibody, cells were washed 3 times in PBS

containing 2 % FBS and incubated for 45 min with the Alexa Fluor 488-labeled anti-mouse antibody from goat as secondary antibody diluted 1 / 250. As negative control yeast cells were incubated only with the secondary antibody. All reactions were performed at room temperature. After washing, cells were fixed in 0.4 % formalin and analyzed by flow cytometry.

Flow cytometry was performed with an FACS Canto equipped with an argon ion laser with an excitation power of 20 mW at 488 nm. Forward and side light scatter were analyzed on linear scales, while fluorescence intensity was determined on logarithmic scales. Analysis gates were set around debris and intact cells on a forward and side light scatter dot plot. The fluorescence histograms of 10,000 cells were generated using gated data. Data analysis was performed using Flowjo 7.5.

3.4 Work with yeast

3.4.1 Yeast strains

All the *C. albicans* strains used in the experiment are listed in Tab.3.5. These were provided by R. Calderone (Georgetown University, USA) and Jesus Pla (Universidad Complutense de Madrid, Spain). In addition, the *S. cerevisiae* strain BY4741 was established in Euroscarf (Johann Wolfgang Goethe-University, Frankfurt am Main).

Tab. 3.5 *C. albicans* strains used in this study

Strains	Synonym	Genotype	Reference
CAF2-1		Δ*ura3*::imm434/URA3	[142]
Δ*chk1*	CHK21	Δ*ura3*::imm434/Δ*ura3*::imm434 Δ*cahk1*::hisG/Δ*cahk1*::hisG-URA3-hisG	[115]
Δ*cek1*	CK43B-16	*ura3/ura3*Δ*cek1::hisG/*Δ*cek1::hisG-URA3-hisG*	[143]
Δ*mkc1*	CM1613	Δ*mkc1*::hisG/Δ*mkc1*::hisG-URA3-hisG	[143]
Δ*hst7*	CDH12	*ura3/ura3* Δ *hst7::hisG/*Δ*hst7::hisG*	[144]
Δ*pbs2*	BRD3	Δ*pbs2*::*cat*/Δ*pbs2*::*cat*-URA3-*cat*	[117]
Δ*hog1*	CNC13	*hog1::hisG/hog1::hisG*-URA3-*hisG* Δ*his1*::hisG/Δ*his1*::hisG	[145]

3.4.2 Preparation of YPD and YP galactose medium

YPD medium (yeast extract peptone dextrose) is a complex culture medium from yeast

extract, peptone and glucose which was used for the cultivation of yeasts. The YPD medium was purchased from Sigma (Y1375) and according to the manufacturer's specifications 50 g of powder were dissolved in 1L MilliQ water. The sterilization of the medium was done as described in 2.3.1.

YP galactose medium is a complex culture medium from yeast extract 10 g, peptone 20 g and galactose 20 g dissolved in 1L MilliQ water. The sterilization of the medium was done as described in 3.2.1

3.4.3 Storage and cultivation

1 ml yeast suspension from the stationary growth phase was stored in cryogenic tubes with YPD medium at -80 °C. For using, a tube was thawed at room temperature and cultivated overnight in an Erlenmeyer flask (100 ml) volume of 20 ml YPD medium at 30 °C with shaking (160 rpm). The next day, the yeast cells were used for an experiment or frozen for new storage.

3.4.4 Labelling yeast with fluorescence dye

The fluorescent labelling of yeast cells was carried out by the coupling of the fluorescein derivative fluorescein isothiocyanate (FITC) to amino groups of cell wall components of yeast. Yeasts were cultured in YPD medium as described in 3.4.3 and the cell number was determined (3.2.3). Subsequently, 1×10^8 yeast cells were transferred into a 1.5 ml reaction tube filled with PBS to 1 ml, harvested by centrifugation (16 000 × g, 5 min, 24 ° C). The supernatant was discarded and the pellet was resuspended in 1 ml PBS. This washing step was repeated twice before the yeasts were added in 1 ml of a dye solution of the dye (Tab. 3.6) and incubated overnight at 4 °C. The next day, the yeasts were washed three times with 1 ml of PBS, either used immediately or stored until use at -20 ° C.

Tab. 3.6 Property of the fluorescence dye

	FITC
Company	Sigma
Article number	F7250
Coupling group	Isothiocyanate
Excitationmax.	495 nm
Emissionsmax	519 nm
Storage	100 mg/ml in DMSO, -20 °C
Final concentration	1.25 mM (500 µg/ml)
Stain buffer	0.1 mM $NaHCO_3$ + 0.5% DMSO, pH 9.0

3.4.5 Genistein- treated C. albicans

From the overnight cultures of yeast, the concentration of yeast suspension was determined. The yeasts were diluted into OD=0.2 in 20 ml YPD. After 2 hours preculture, the yeasts were diluted into OD=0.1 and different concentration of genistein were added for another 24 hours. The yeasts were then prepared for further experiments.

3.4.6 Growth curve of yeast

The investigation of the growth of yeast was based on the measurement of optical density (OD) of yeast suspensions using a multi-well photometer. The yeasts from an overnight culture were transferred (180 µl / well) into a column in a 96-well culture plate and measured at 620 nm. This preculture was diluted to an optical density (620 nm) of 0.2, for 2 h growth and started with OD=0.1 for working culture with or without genistein 100 µM treatment in YP galactose medium and determined the OD hourly by the microtiter plate reader µQuant.

3.4.7 Measurement of oxygen consumption

The test organisms *S. cerevisiae* BY4741 and *C. albicans* CAF2-1 were grown in YPD medium overnight at 30 °C on a shaker. The culture was diluted into OD=0.2 in 20 ml YP galactose. After 2-3 hours preculture, this culture was then diluted to an optical density (620 nm) of 0.15 (for *S. cerevisiae*) and 0.05 (for *C. albicans*) determined by the microtiter plate reader

µQuant.

Oxygen consumption was measured by a fluorometric assay as previously described [146]. Round-bottomed OxoPlates® (PreSens, Regensburg, Germany) were used instead of the clear polystyrene microtiter plates. In a 96 well round-bottomed OxoPlates® (PreSens, Regensburg, Germany) (Greiner, Recklinghausen, Germany), compounds were serially diluted by a factor of 3. In the first column 30 µl of the working solution was added to 60 µl YP galactose medium; the other columns contained 60 µl medium to which 30 µl was added from the preceding column. By this procedure not only the compounds were serially diluted but also the amount of solvent, which was still 33% in the first column at this stage. Finally, for inoculation 120 µl of the diluted cell suspension was added. For *C. albicans*, all experiments were started with OD=0.05; for *S .cerevisiae*, OD started with 0.15. The plates were then incubated at 30 °C and 650 rpm using the Heidolph instruments Titramax 1000 shaker.

The determination was done as described by the manufacturer i.e. fluorescence of the indicator and the reference dye was determined with a fluorescence microtiter plate reader (Cytofluor, Reader Series 4000, PerSeptive Biosystems) from the bottom using the excitation wavelength λ_{ex} 530 nm and the emission wavelength λ_{em} 620 nm for the indicator and λ_{ex} 530 nm / λ_{em} 590 nm for the reference dye. Calibration was done with water saturated with air (100%) and with an aqueous Na_2SO_3 solution 1 g / 100 ml (0%) according to the supplier's protocol.

3.4.8 Measurement of ROS accumulation

Endogenous amounts of ROS in yeasts were measured by a fluorometric assay using H_2DCFDA, SE (Invitrogen, catalog number D-2935) as a ROS indicator as previously described [147]. The succinimidyl ester of 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), is a chemically reduced and acetylated form of the highly fluorescent dye 2',7'-dichlorofluorescein (DCF) (Fig. 3.5).

Logarithmically growing *C. albicans* cells were suspended in sterile water to OD=0.5. Then H_2DCFDA, SE was added from a stock solution of 2 mg/ml in DMSO to a final concentration of 40µg/ml. After 30 min of incubation at 30 °C, the cells were collected by centrifugation and resuspended in sterile water. Then 120 µl of this mixture were added to a dilution series of genistein, rotenone and antimycin in a 96 well microtiter plate. The development of fluorescence was monitored after 2 hours at 30 °C at λex=485 nm and λem=535 nm through the bottom of the plates by a fluorescence multi-well plate reader.

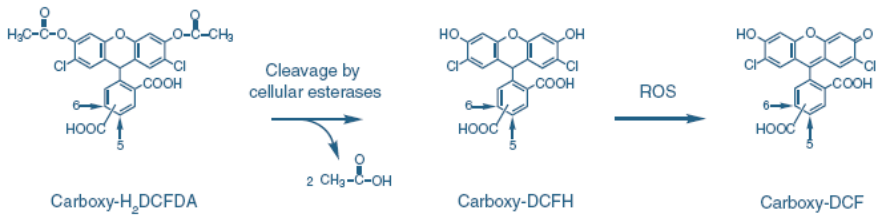


Fig. 3.5 Principle of the detection: Nonfluorescent 5-(and-6)-carboxy-2‘,7‘-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) permeates live cells and is deacetylated by nonspecific intracellular esterases. In the presence of ROS, which are produced throughout the cell, particularly during oxidative stress, the reduced fluorescein compound is oxidized and emits bright green fluorescence.

3.4.9 Spectrophotometric assay of complex I activity with *C. albicans*

From overnight cultures of *C. albicans*, precultures were prepared by diluting the overnight culture to an optical density (OD at 620nm) of 0.2 determined with the µQuant microtiter plate reader by taking 180 µl cell suspensions from overnight culture. This culture was allowed to grow for 2-3 hours until the exponential growth phase was reached. Then working cultures were prepared by diluting the pre-culture to OD_{620nm} = 0.1, and adding of 12.3 µl of either DMSO or genistein and rotenone from the 200 mM stock solution, so that the final concentration of genistein was 123 µM, the final concentration of rotenone was 63.3 µM. The samples were taken after 3 hours by centrifugation. OD was measured in parallel. The supernant was discarded and the pellet was washed carefully with PBS at pH=7 for 2 times. The pellet was then suspended in 1 ml PBS.

To assay the redox activity of the complex I within the microorganisms, in the normal 96 well plate 20 µl from the yeast suspension were added in the first 3 wells of the plate and then 160 µl of DCIP (dichlorophenolindophenol) (D1878, Sigma) with 40 µM in PBS at pH 7 was added. Measurement of the change in the DCIP color at different times was carried out at 600 nm with the microtiter plate reader µQuant.

3.4.10 Ethanol determination

From overnight cultures of *C. albicans* and *S. cerevisiae* precultures were prepared by diluting the overnight culture to an optical density (OD at 620nm) of 0.2 determined with the µQuant microtiter plate reader, using 180 µl cell suspensions in 20 mL YPD. This culture was allowed to grow for 2-3 hours until the exponential growth phase was reached. Then working cultures were prepared by diluting the pre-culture to OD_{620} = 0.1 and 10 µl of either DMSO or

genistein from the 200 mM stock solution were added so that the final concentration of genistein was 100 µM. For ethanol determination 200 µL were taken as sample from the culture every hour and immediately centrifuged at 14,000 rpm and 4 °C for 10 minutes. The supernatants were stored in liquid nitrogen until ethanol was determined.

Ethanol was determined as described previously [148]: All solutions of the enzyme mix were prepared in 100 mM NaPP buffer, or which 4.46 g were solved in 100 ml water. The pH was adjusted with phosphoric acid. The enzyme mix comprised alcohol dehydrogenase (ADH; 288 U / mg; Fluka; stock solution: 3 U / µl NaPP, pH 7.5) and NAD^+ (Biomol; stock solution: 11 Mm in NaPP, pH 8.5). For the preparation of the enzyme mix, 33 µl ADH were added to 5 ml NAD^+ solution. Ethanol standards (50 mM) were prepared in YPD. In each well, 5 µl of the sample or the standard solution was placed, and the reaction was started by the addition of 45 µl of the enzyme mix. After incubation at room temperature for 30 min, the absorbance at 340 nm was determined with the microtiter plate reader (µQuant, Biotek). Four aliquots of each sample were analyzed.

3.4.11 Flow cytometric analysis of cell wall glucans and mannans

3.4.11.1 β-(1,3)-glucans analysis

The accessibility of β-1, 3-glucans on the yeast cell wall was investigated by flow cytometry. The methods were performed according to Jouault et al [149]. β-1, 3-glucans were stained by a monoclonal antibody: 1 ml of 10^6 cells / ml were washed with PBS containing 2 % FBS and then incubated for 1 hour with 200 µl of the antibody solution which was prepared by an 1 / 150 dilution of the delivered antibody. For detection of the primary antibody, cells were washed 3 times in PBS containing 2 % FBS and incubated for 45 min with the Alexa Fluor 488-labeled anti-mouse antibody from goat as secondary antibody diluted 1 / 250. As negative control yeast cells were incubated only with the secondary antibody. All reactions were performed at room temperature. After washing, cells were fixed in 0.4 % formalin and analyzed by flow cytometry.

Flow cytometry was performed with an FACS Canto equipped with an argon ion laser with an excitation power of 20 mW at 488 nm. Forward and side light scatter were analyzed on linear scales, while fluorescence intensity was determined on logarithmic scales. Analysis gates were set around debris and intact cells on a forward and side light scatter dot plot. The fluorescence histograms of 10,000 cells were generated using gated data. Data analysis was performed using Flowjo 7.5.

3.4.11.2 Mannan analysis

The accessibility of mannan on the yeast cell wall was investigated by flow cytometry. Staining of the yeasts with FITC-labeled concanavalin A, which binds to carbohydrates, were performed according to Tkacz et al [150]. Yeast from an overnight culture was adjusted to a final optical density of 0.1 at 620 nm in 0.5 ml. The cells were washed twice with 0.9 % NaCl and collected by centrifugation and resuspended in 0.5 ml of the FITC-concanavalin A (stock solution with 1 mg/ml) diluted 1: 50 with 0.9 % NaCl. After 30min at room temperature, the cells were again collected by centrifugation and washed twice with 1 ml 0.9 % NaCl. All reactions were performed at room temperature. After washing, cells were fixed in 0.4 % formalin and analyzed by flow cytometry.

Flow cytometry was performed with an FACS Canto equipped with an argon ion laser with an excitation power of 20 mW at 488 nm. Forward and side light scatter were analyzed on linear scales, while fluorescence intensity was determined on logarithmic scales. Analysis gates were set around debris and intact cells on a forward and side light scatter dot plot. The fluorescence histograms of 10,000 cells were generated using gated data. Data analysis was performed using Flowjo 7.5 software.

3.4.12 Cell surface hydrophobicity (CSH) test

CSH was measured by the biphasic separation method (Microbial Adhesion To Hydrocarbons) as described previously [151]. For each strain tested, 1.5 ml of yeast suspension (OD_0=1) was placed into a clean glass tube and overlaid with 1 ml hexadecane (Sigma-Aldrich : 52210). The samples were vortexed for 2 min and settled down for 10 min, the phases were allowed to separate. The OD 620nm was measured for the aqueous phase. Genistein treated yeasts were prepared as shown in (0).

The Hydrophobicicty was determined with the help of the following formula:

Hydrophobicity index= (OD_0-OD_{620nm} after hexadecane overlay)/ OD_0×100 .

3.5 Infection

3.5.1 Phagocytosis assay

Phagocytosis of *C. albicans* by macrophages was conducted using a fluorimetric phagocytosis test. It was quantified as described [152]. 100 µl of 2×10^6 macrophages/ml were seeded in each well of 96 well microtiter plates followed by incubation for 2 hours to let the cells

adhere to the plates. Macrophages were pre-incubated for 2 hour with the test compounds, before FITC-stained *C. albicans* was added. The ratio of macrophages: yeast was 1 : 2. For 24 hours genistein treatment 1×10^6 macrophages / ml were used. For 48hours genistein treatment of macrophages the cell concentration was 0.5×10^6 macrophages / ml. In this way, there are enough cells for the phagocytosis assay. Phagocytosis was allowed to proceed at 37 °C in 10 % CO_2 for different periods of time. The medium was removed and 100 µl trypan blue (Fluka, 250 µl / ml in PBS) were added to quench the fluorescence of yeasts which were not internalized. After an incubation of 1 min at room temperature, the trypan blue solution was removed. The number of internalized yeasts was estimated from fluorescence measurements (λ_{Ex}=480 nm and $\lambda_{Em=}$ 520 nm) through the bottom of the plates by a fluorescence multi-well plate reader.

3.5.2 Cytokine detection by ELISA

3.5.2.1 Samples preparation

The cells were harvested and counted under normal culture condition. 100 µl of 3×10^5 macrophages / ml were seeded in each well of 96 well microtiter plates and allowed to adhere for 2 hours. 1×10^8 *C. albicans* / ml were harvested from culture with or without genistein by centrifugation (13,000 rpm, 5 min, 24 °C), and washed twice with 1 ml PBS. They were suspended in DMEM cell culture medium. The supernatant was removed from the cell culture and 100 µl of the yeast suspension were added. When macrophages were treated with genistein, the compound was added 1 hour before the yeast was added. The ratio of macrophages: yeast was 1:10 in all experiments. TNF-α concentrations were determined after yeast–macrophage incubation time of 1 hour, IL-10 concentrations were determined after 5 hours. The supernatant was collected by centrifugation (3000 rpm 5 min 4 °C). These samples were analyzed either immediately by ELISA or stored in -80°C. The detection was done as described in (0).

4 RESULTS

4.1 Effect of genistein on the function of RAW264.7 macrophages

4.1.1 Cell viability

Genistein is known to inhibit cell proliferation of human breast cancer cells (MCF-7), human ovarian cancer cells (HeLa), and human prostate cancer cells (PC3) [153-155]. We tested the influence of genistein concentrations and incubation times on the viability of the macrophages by the WST-1 assay.

The results showed that genistein has an inhibitory effect on the cell viability in a time- and dose-dependent manner (Fig. 4.1). When the macrophages were treated with genistein for the short time of only 2 h, none of the genistein concentrations led to decreased signals. However, when the incubation time was extended to 24 and 48 hours, genistein concentrations of 100 - 50 µM reduced the cell viability to 70 % - 80 % and 50 % - 60 % after 24 and 48 hours treatment ($P < 0.01$), respectively. With lower genistein concentrations, no influence on the cell viability was observed.

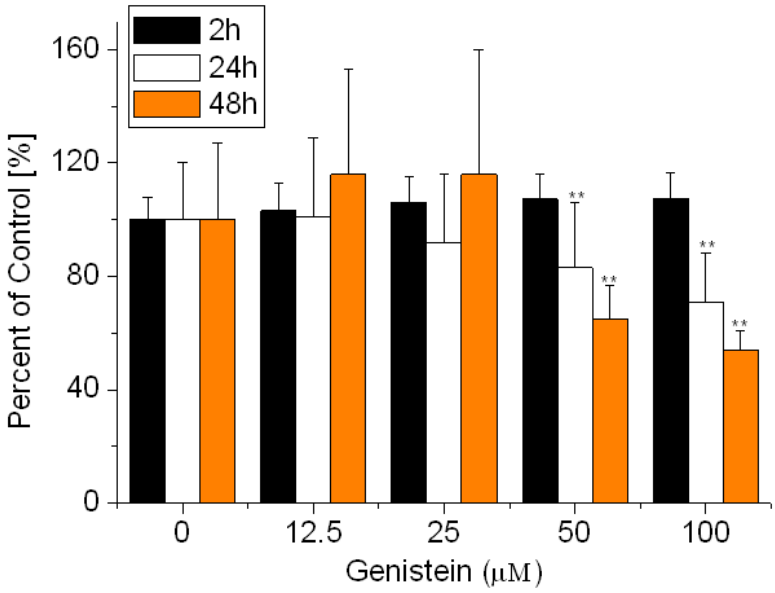


Fig. 4.1 Effects of genistein on the viability of macrophages determined by the WST-1 assay. Data are given with respect to the solvent control (100 %) and are the average of three independent experiments.
**: $p<0.01$ compared with solvent control.

4.1.2 Cell cycle

Genistein was described to arrest the cell cycle of human gastric cancer cells (HGC-27) and human breast adeno-carcinoma cells (MCF-7), as well as neuronal cells at the G2/M phase [156-159]. To elucidate whether the reduction of signals in the WST-1 test was due to a decreased proliferation rate, which could be caused by a cell cycle arrest, the influence of genistein on the cell cycle of macrophages was investigated by flow cytometry.

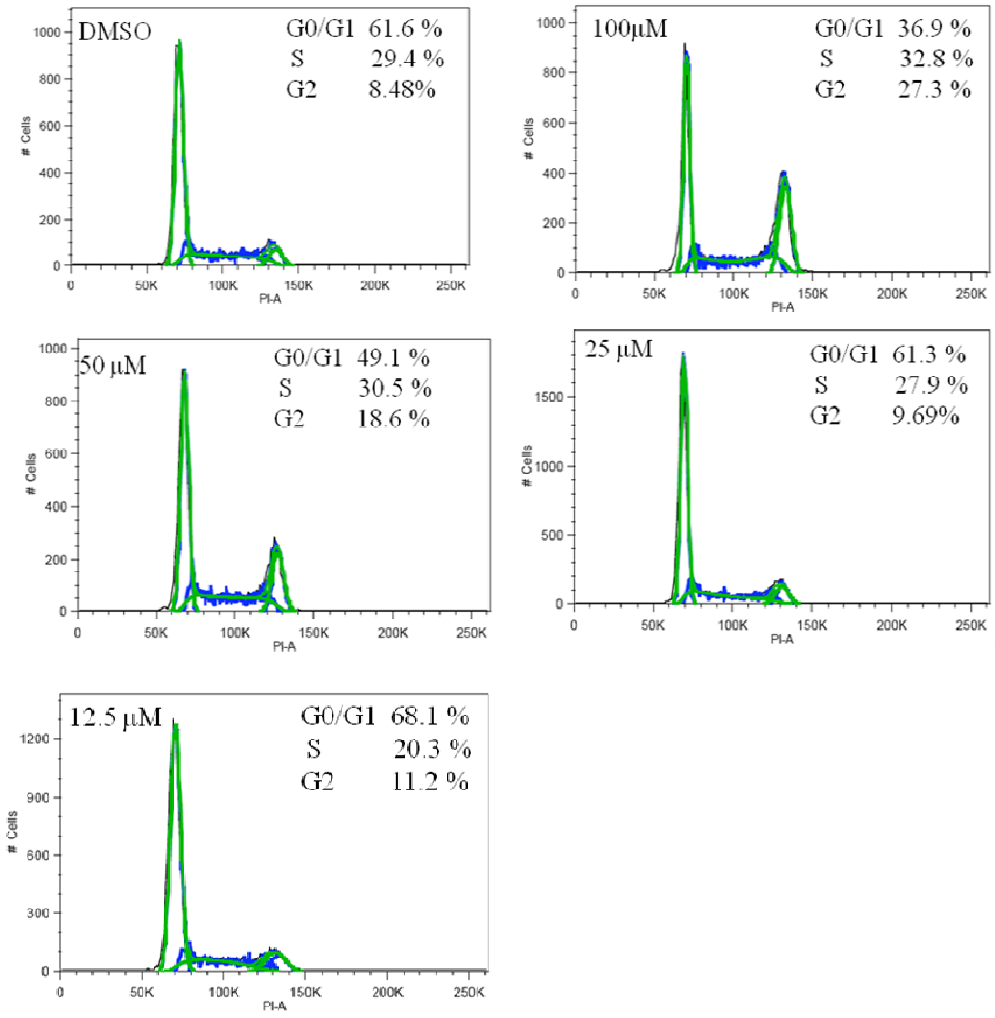


Fig. 4.2 The cell cycle distribution of macrophages after treatment with genistein for 24 hours. The cell cycle distribution was determined by flow cytometry after PI staining. All experiments were performed in duplicate and gave similar results.

As shown in Fig. 4.2, genistein treatment induced a significant accumulation of cells in the G2/M phase of the cell cycle in a concentration- and time-dependent manner, with a parallel depletion of the percentage of cells in G0/G1. When the macrophages were treated with genistein for 24 hours, the percentage of cells in S phase was between 20 - 30%, independent on the

genistein concentration. However, the number of cells in the G2/M phase increased significantly from 8.5% to 27.3% and 18.6%, for 100 and 50 µM genistein, respectively. The numbers in the G0/G1 phase decreased from 61.6 % to 36.9 % and 49.1 % respectively.

The effect was more striking when the incubation time was extended to 48 hours, which also influenced the amount of cells in the S phase (Fig. 4.3): the amount of cells in the S phase increased from 15.8 % to 29.7 % and 19.5 %, respectively. The percentage of cells in the G2 / M phase increased from 11.4 % to 51.9 % and 30 %, respectively, whereas the percentage in the G0 / G1 phase decreased only slightly for 50 µM genistein from 67.2 % to 51.8 % but significantly to 9.2 %, when 100 µM genistein was used. Lower concentrations of genistein had no obvious effect on the cell cycle of the cells.

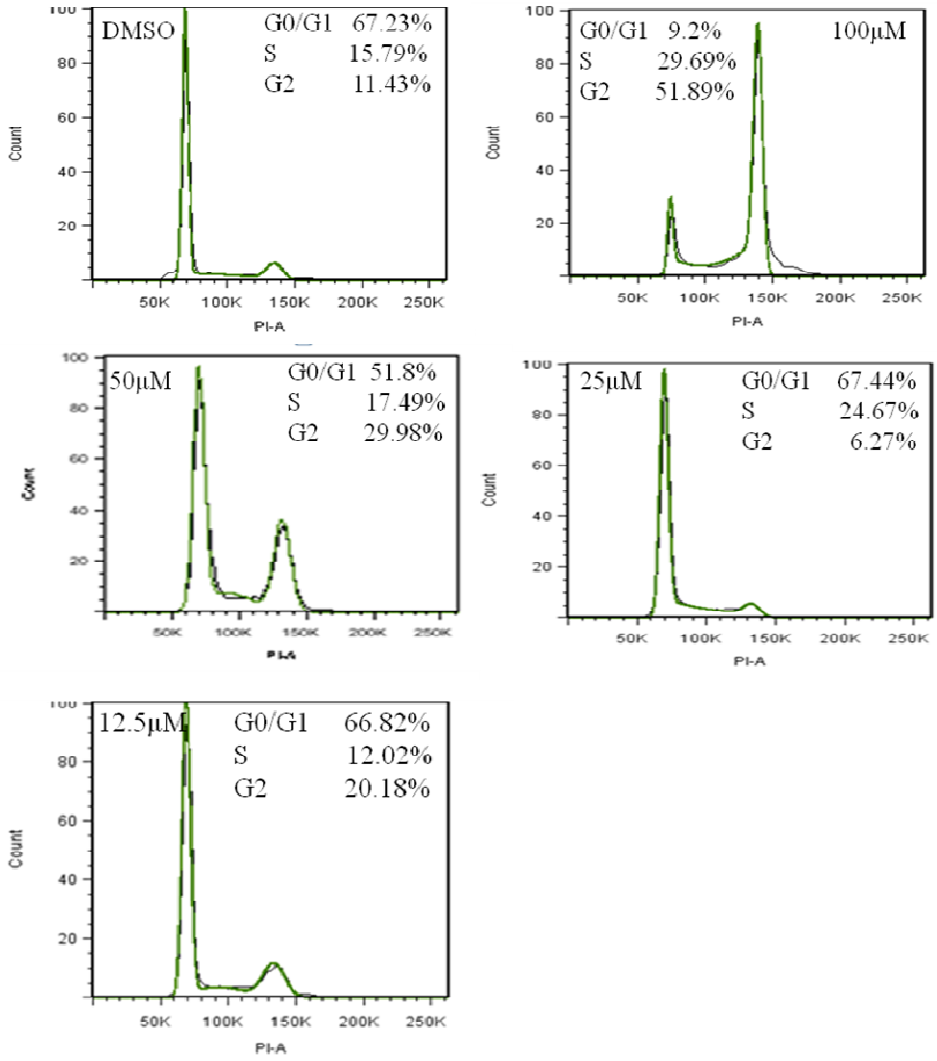


Fig. 4.3 The cell cycle distribution of macrophages after treatment with genistein for 48 hours. The cell cycle distribution was determined by flow cytometry after PI staining. All experiments were performed in duplicate and gave similar results.

4.1.3 Morphology

Microscopic observations revealed that genistein influenced the morphology of the RAW264.7 macrophages. Thus, pictures were taken with an inverted contrast microscope after treatment of the cells with genistein for 2, 24, and 48 hour. Aberrations in cellular morphology were observed in a time- and dose-dependent manner (Fig. 4.4). After 2 hours of treatment, the cells were round and well attached to the bottom of the plates. The different concentrations of genistein exerted no effects on the morphology. After 24h, the cells were confluent and round in the control culture; whereas, in the genistein-treated culture, the cells were well attached, but the cell size increased and showed irregular shapes such as diamond shapes or pseudopodia-like protrusions. After 48h, these morphological changes became even more obvious.

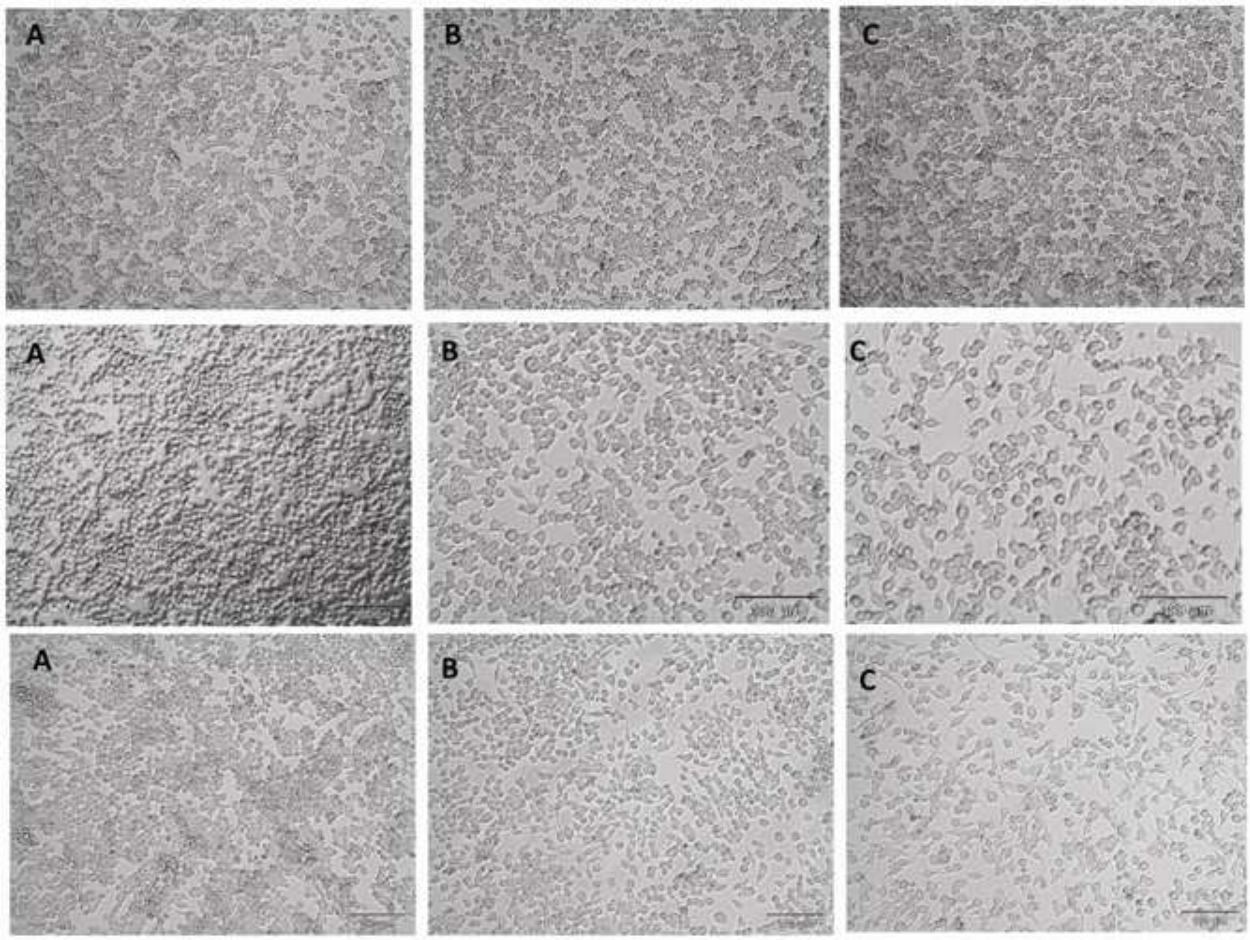


Fig. 4.4 Morphological change of macrophages RAW264.7 after treatment with genistein. Different concentrations of genistein were incubated for 2 h (Top), 24h (Middle) and 48h (Bottom); (A) Control, (B) 12.5µM, (C) 25µM. The pictures were taken under the inverted contrast microscope. They are representatives of three independent experiments which were repeated on different days.

4.1.4 F-actin cytoskeleton

The cytoskeleton of cells consists of highly dynamic actin polymer structures that play a critical role to maintain cellular morphology. As genistein significantly altered the morphology of macrophage cells (Fig. 4.4), we examine the effects of genistein on the cytoskeleton network in RAW264.7 macrophages.

Cytochalasin B is a well known cytoskeleton inhibitor, which blocks actin polymerization by preventing the binding of monomeric G-actin to the growing (+) end of actin filaments (F-actin) [160]. Cytochalasin B also inhibits cell division by destruction of the contractile ring [161]. As it is shown in Fig. 4.5, there is clear difference between cytochalasin B-treated (Fig. 4.5 B) and untreated (Fig. 4.5 A) cells. Cytochalasin B-treated cells were round and had three nuclei, and the filament structures were not visible; whereas the untreated cells were round and the actin filaments were clearly discernible at the edges of the cells. After genistein treatment of the macrophages, changes of the F-actin structure were obviously induced: (i) the cell size increased and the cells had two nuclei, a long pseudopodia-like protrusion was formed after treatment with 100 μM genistein; (ii) lobopodia-like protrusions were formed after treatment with 50 μM genistein; (iii) whereas macrophage treated with 25 μM genistein showed a typical spindle morphology, the actin was clearly stained at the edges of the cells and had long pseudopodia-like protrusions. Macrophage treated with 12.5 μM genistein showed similar effect.

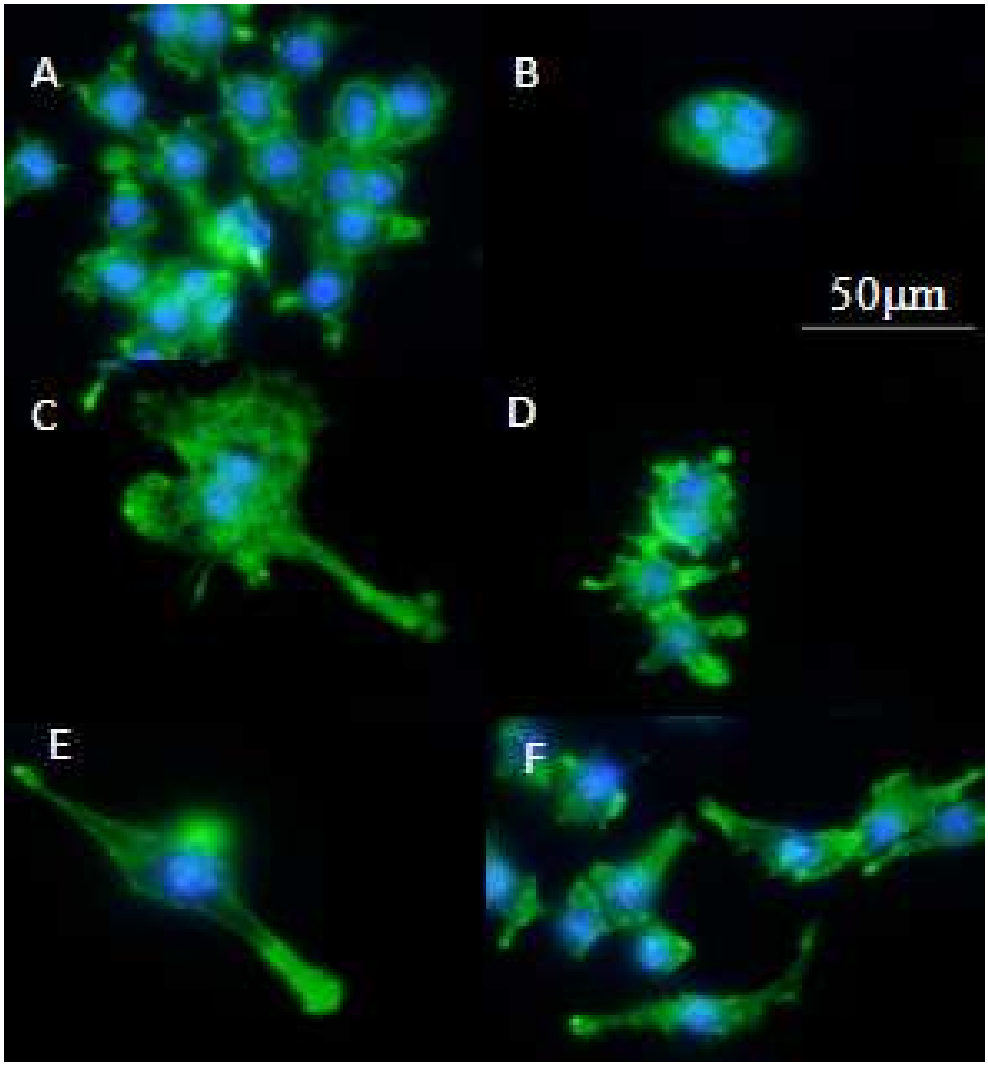


Fig. 4.5 Effect of cytochalasin B and genistein on the actin cytoskeleton of macrophage RAW 264.7. The cells were incubated with cytochalasin B 2 nM (B) and genistein (C – F) for 48 hours, the actin filaments were stained with Alex488-phalloidin (Green fluorescence) and the cell nuclei were stained with DAPI (Blue Fluorescence), the pictures are the overlay of both fluorescence channels. (A) DMSO; (B) Cytochalasin B 2nM; (C) Genistein 100µM; (D) Genistein 50µM ; (E) Genistein 25µM; (F) Genistein 12.5 µM.

4.1.5 Nitric oxide production of macrophages stimulated by LPS

One of the major functions of macrophages is the production of signalling compounds and of reactive oxygen and nitrogen (nitric oxide, NO) species to kill foreign material. Thus, the effects of genistein on NO synthesis in RAW264.7 macrophages were investigated. The macrophages were stimulated with LPS and genistein for 20h. The NO production was determined via the nitrite concentrations in the culture medium using the Griess reaction. As shown in Fig. 4.6, cells treated with LPS for 20 h produced high amounts of nitrite (32±4 µM), which were 10-fold higher than the basal production of the unstimulated cells (3±1 µM). A

marked reduction of nitrite production was observed after genistein treatment (12.5 - 100μM). Cell viability of the macrophages stimulated with LPS and genistein was analyzed with the WST-1 assay to exclude the possibility that the inhibitory effect of genistein was due to cytoxic effects (Fig. 4.6). The viability of all cultures was not affected by any of the treatments and generally was > 90%. This result indicated that the inhibition of nitrite production by genistein was not due to cell death.

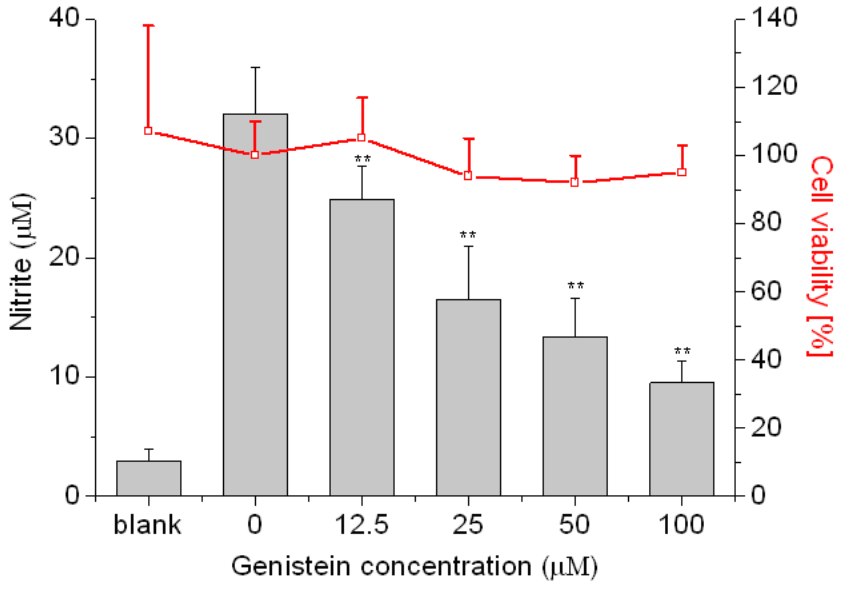


Fig. 4.6 Influence of genistein on the NO production and viability of LPS stimulated RAW264.7 macrophages. Left Y-axis: nitrite concentrations in the cell culture supernatants of LPS treated macrophages in the presence or absence of different concentrations of genistein. Results are expressed as means ± SD; n=8. Right Y-axis: Cell viability of LPS treated macrophages in the presence and absence of genistein.The cell viability of the solvent control (0 μM genistein) was 100 %. Results are expressed as mean ± SD; n=8.
**: $p<0.01$ compared with control.

4.1.6 Nitric oxide production of macrophages stimulated by IFN-γ and IFN-γ+ LPS

IFN-γ exhibits a wide spectrum of biological activities, for example antiviral immunomodulatory effect. Not only the endotoxin LPS but also cytokines, such as IFN-γ, stimulate macrophages to produce NO. A combination of IFN-γ plus LPS is usually used to achieve the maximal induction of NO in macrophages. Therefore, we determined the genistein effect on the macrophages stimulated with (i) IFN-γ and (ii) IFN-γ plus LPS for 20 h. As shown in Fig. 4.7, genistein led to a dose-dependent inhibition of nitrite accumulation by IFN-γ alone and in combination with LPS. Cells treated with IFN-γ / IFN-γ+ LPS for 20 h produced a high level of nitrite (30±2 μM, 52±2 μM), which was significantly higher than the basal production of

the unstimulated cells (0 μM). A marked reduction of nitrite production was observed after genistein treatment (4 - 111 μM genistein for IFN-γ, 37 - 111 μM genistein for IFN-γ + LPS).

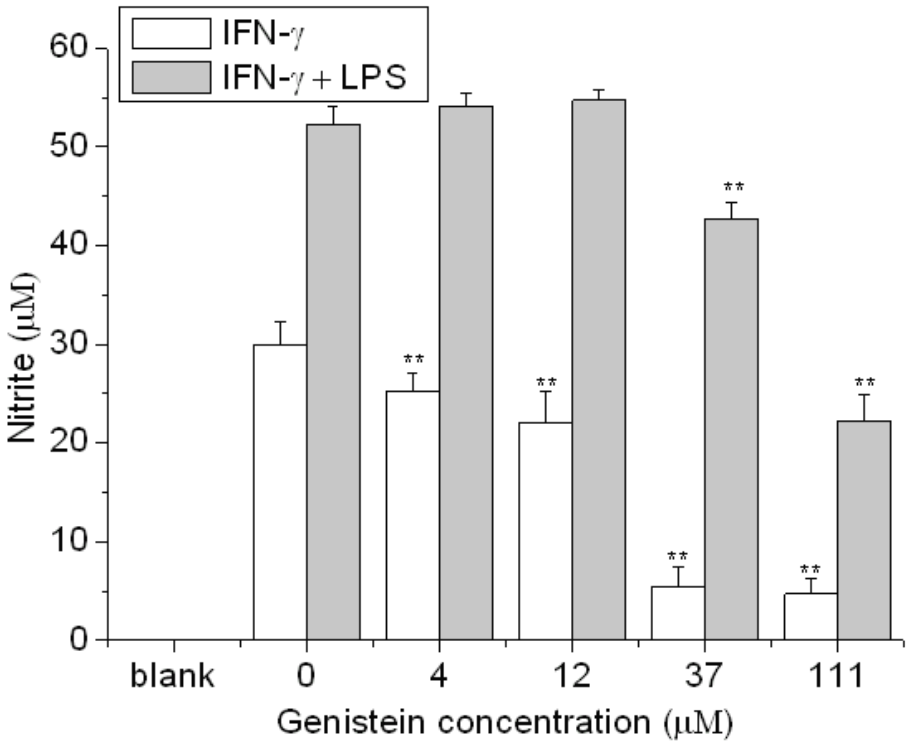


Fig. 4.7 Influence of genistein on the NO production of IFN-γ and LPS+IFN-γ stimulated RAW264.7 macrophages. Nitrite concentrations in the cell culture supernatants of IFN-γ and LPS+IFN-γ treated macrophages in the presence or absence of different concentrations of genistein. Results are expressed as mean± SD; n=8.
**: $p<0.01$ compared with control.

4.1.7 Cytokine production of macrophages stimulated by LPS

The effects of genistein on the production of the cytokines TNF-α, IL-6, and IL-10 by RAW264.7 macrophages were investigated. As shown in Fig. 4.8, genistein showed a dose-dependent inhibition of the cytokine accumulation in the supernatants of the LPS activated macrophages. After 1.5 h of stimulation, LPS-treated cells produced a high level of TNF-α (1100±50 pg/ml), which were 5-fold higher than the basal production (200±20 pg/ml). This LPS-induced TNF-α production could be significantly reduced by incubation with the highest genistein concentration of 100 μM. After 5 hour of stimulation, the induction of IL-6 secretion by LPS could also be detected (390±26 pg/ml in comparison to 0 pg/ml of untreated cells). Even the lowest concentration of genistein (3.7 μM) resulted in a significant reduction of the IL-6 production. After 6 hours, high levels of IL-10 (5190±260 pg/ml) were measured (unstimulated cells: 1130±503 pg/ml), which were reduced by elevated genistein concentrations of 33-100 μM.

Cell viability of the macrophages stimulated with LPS and genistein for 6 hours was analyzed to exclude the possibility that the inhibitory effect of genistein was due to cytoxicity. The viability of all cultures was not affected by any of the treatments and generally was > 80%. This result indicated that the inhibition of nitrite production by genistein was not due to cell death.

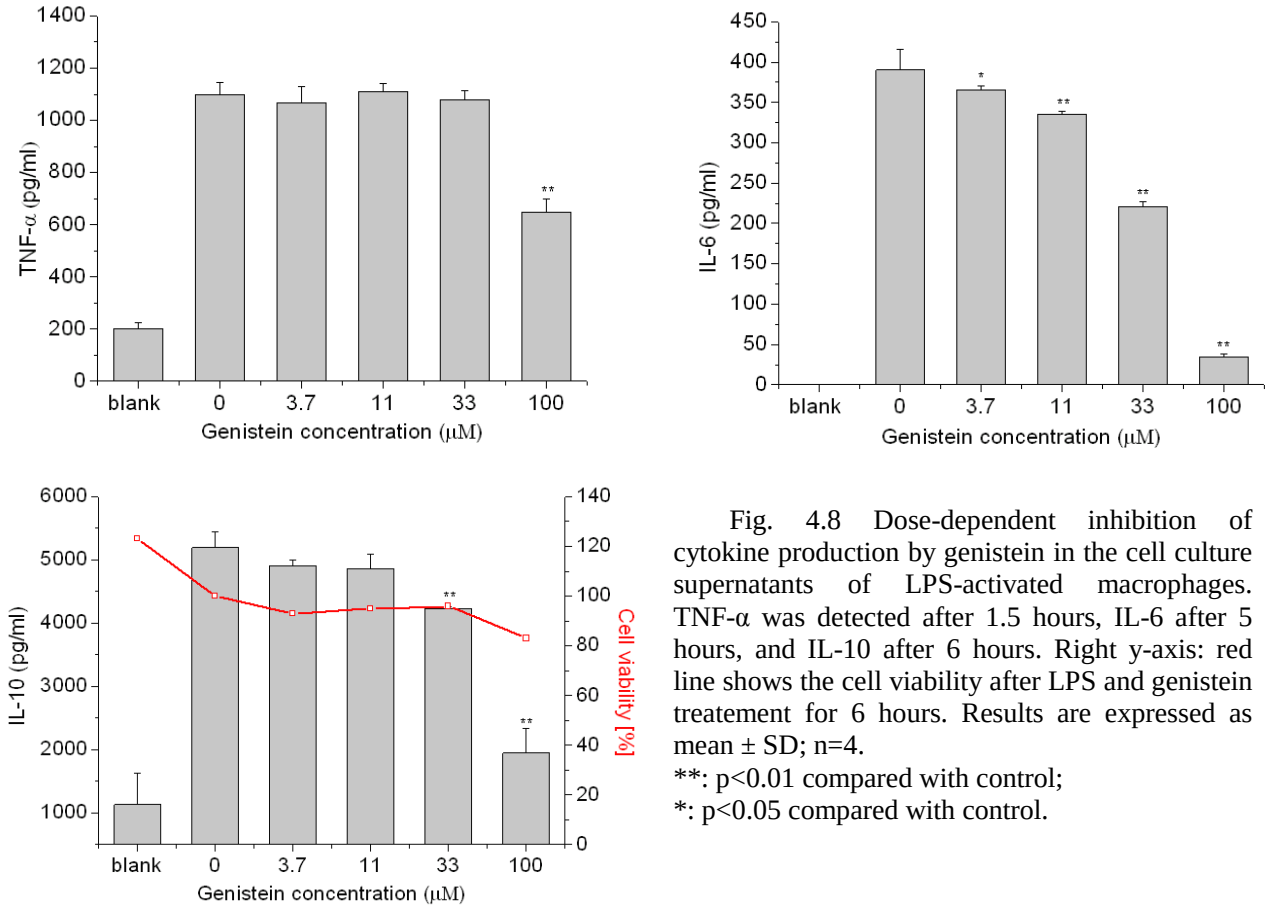


Fig. 4.8 Dose-dependent inhibition of cytokine production by genistein in the cell culture supernatants of LPS-activated macrophages. TNF-α was detected after 1.5 hours, IL-6 after 5 hours, and IL-10 after 6 hours. Right y-axis: red line shows the cell viability after LPS and genistein treatement for 6 hours. Results are expressed as mean ± SD; n=4.
**: $p<0.01$ compared with control;
*: $p<0.05$ compared with control.

4.1.8 Specific kinase inhibitor involved in LPS and IFN-γ pathway

4.1.8.1 NO production

Genistein inhibits the NO production induced by LPS and IFN-γ as well as by the combination of LPS + IFN-γ. Genistein is, in addition, a well known tyrosine kinase inhibitor. Therefore, we examined which tyrosine kinases and downstream kinases are involved in this process. We found that tyrosine kinases Syk, Jak2, Src, PI3Kinase, and Akt kinase are involved in the LPS-induced NO production in a concentration-dependent manner; whereas, tyrosine kinases (Syk and Jak2, but not Src), PI3 kinase, and Akt kinase are involved in the IFN-γ-induced NO production in a concentration-dependent manner (Tab.4.1).

Tab. 4.1 Effects of specific kinase inhibitors in NO production by LPS, IFN-γ and LPS+ IFN-γ stimulated RAW264.7 macrophages.

Target kinase	Inhibitor	LPS	IFN-γ	LPS+ IFN-γ
Jak2	AG490	0.16-340 μM	4.2-340 μM	12.6-340 μM
Src	PP2	3.7-11.1 μM	----	11.1μM
Syk	Syk inhibitor	4.7-14 μM	4.7-14 μM	4.7-14 μM
MEK1/2	PD98059	----	----	----
P38	SB203580	26.7μM	----	----
PI3K	LY294002	0.82-22.2 μM	7.4-22.2 μM	7.4-22.2 μM
PI3K	Wortmanin	2.6-7.8 μM	2.6-7.8 μM	2.6-7.8 μM
AKT	Akt1/2 inhibitor	0.13-33.3 μM	33.3 μM	11.1-33.3 μM
PTP	Bpv(phen)	----	----	----

4.1.8.2 Cytokine production

As shown in Tab.4.2, specific kinase inhibitors showed a dose-dependent inhibition of the cytokine (TNF-α, IL-6, and IL-10) accumulation in the supernatants of LPS-activated macrophages. Tyrosine kinases (Syk, Jak2, and Src), MAP kinase, PI3 kinase, Akt kinase, and protein phosphotyrosine phosphatase (PTP) are involved in the production of cytokine IL-6, IL-10, and TNF-α.

Tab. 4.2 Specific kinase inhibitors effect on the cytokine productions in the cell culture supernatants of LPS-activated macrophages. TNF-α was detected after 1.5 hours, IL-6 after 5 hours, and IL-10 after 6 hours.

Target kinase	Inhibitor	IL-6	IL-10	TNF-α
Jak2	AG490	12-37 μM	12-37 μM	12-37 μM
Src	PP2	0.4-11.1 μM	0.4-11.1 μM	1.2-33 μM
Syk	Syk inhibitor	1.6-14 μM	1.6-14 μM	1.6-14 μM
MEK1/2	PD98059	180 μM	6.7-180 μM	6.7-180 μM
P38	SB203580	2.2-20 μM	2.2-20 μM	2.2-20 μM
PI3K	LY294002	0.27-22.2 μM	0.27-22.2 μM	0.27-22.2μM
PI3K	Wortmanin	0.013-7.8 μM	0.013-7.8 μM	1.3-7.8 μM
AKT	Akt1/2 inhibitor	0.4-33.3 μM	0.4-33.3 μM	0.4-33.3 μM
PTP	Bpv(phen)	1.6-14 μM	1.6-14 μM	4.7-14 μM

Note: The concentration ranges, shown in the Tab.4.1 and Tab.4.2, led to a strong inhibition of NO and cytokine production to less than 70 % compared with solvent control, but had no influence of the cell viability.

4.1.9 Influence of genistein on the LPS-induced MAP kinase phosphorylation

MAP kinase pathways are involved in the LPS-induced macrophage activation [162]. To further delineate and confirm the signalling process involving MAP kinases in the regulation of LPS-induced activation of macrophages by genistein, we evaluated the induction of their phosphorylation by performing western blot analysis.

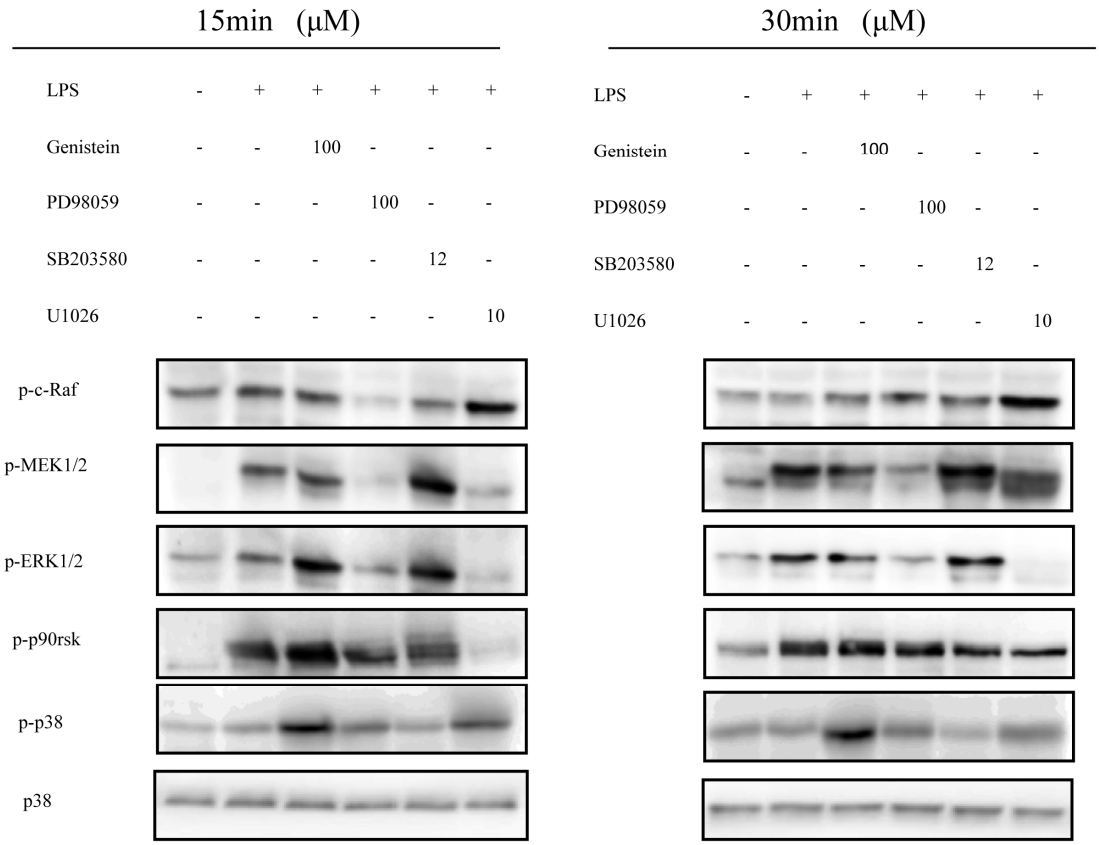


Fig. 4.9 Determination of the activation of the MAP Kinase signalling cascade in lysis of macrophages exposed to LPS and genistein for different time intervals. Macrophages were pretreated with different inhibitors for 1 hour and then stimulated with LPS in the presence of inhibitors for 15 min and 30 min. Western blot analysis of p-Raf, p-MEK1/2, p-ERK1/2, p-p90rsk, p-p38, and p38.

As shown in Fig. 4.9, phosphorylation of Raf, MEK1/2, and ERK1/2 after LPS treatment was detectable after 15 minutes post-stimulation and sustained until 30 minutes. Phosphorylation of p38 after LPS treatment is not as obvious as p-ERK1/2. However, genistein together with LPS showed a strong phosphorylation of ERK1/2, the downstream protein p90rsk, as well as p38. Using specific kinase inhibitors as control, the results showed that the specific MEK1/2 kinase inhibitors U1026 and PD98059 significantly inhibited the phosphorylation of MEK1/2 and

ERK1/2, and slightly increased p38 phosphorylation; while the specific kinase inhibitor for p38 (SB203580) inhibited the phosphorylation of p38 and increases MEK/ERK1/2 phosphorylation.

4.1.10 NF-κB and AP-1 activation detected with RAW-Blue™ Cells

RAW-Blue™ cells are derived from RAW264.7 macrophages. They are stably transfected to express a secreted embryonic alkaline phosphatase (SEAP) gene inducible by NF-κB and AP-1 transcription factors, so that the gene product SEAP is detectable in the supernatant upon TLR, RLR, or NOD stimulation. LPS is a standard stimulus for the TLR-4 receptor pathway. Thus, RAW-Blue™ macrophages were treated with LPS and genistein for 20h and the activity of SEAP was detected. LPS showed a strong activation of NF-κB and AP-1 as shown in Fig. 4.10. Genistein caused a dose-dependent inhibtory effect on the activation of NF-κB and AP-1. A strong inhibition of NF-κB and AP-1 activation was observed for the highest genistein concentration of 100 µM ($p<0.01$); whereas, at lower concentrations effects were less pronounced (33 µM genistein inhibitory effect with $p<0.05$). The cell viability result shows that the inhibitory effect is not due to toxicity effects.

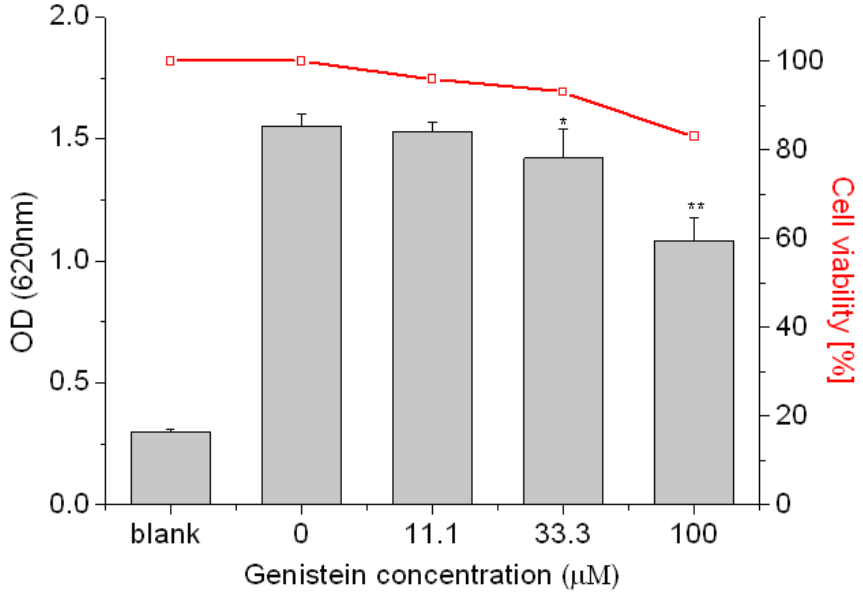


Fig. 4.10 Inhibitory effect of genistein on NF-κB and AP-1 activation of RAW-Blue™ cells by LPS. Right Y-axis: Cell viability of LPS treated RAW-Blue™ cells in the presence and absence of genistein.The cell viability of the solvent control (0 µM genistein) was 100 %. Results are expressed as means ± SD; n=4.
**: $p<0.01$ compared with control; *: $p<0.05$ compared with control.

4.1.11 Gene expression related to Toll-like receptor pathways

In order to confirm the genistein effect on the macrophages in the transcriptional level, we used a Toll-like receptor pathway PCR array to analyze the expression of related genes after treatments with (i) LPS and (ii) LPS together with genistein. A significant number of genes were differentially regulated compared to control cells (Fig. 4.11) (Appendix). Genistein alone could up-regulate the genes CD80, MEKK1, c-fos, Rela, and Ticam2. LPS could up-regulate 20 genes including (a) cytokines IFN-β, IL-10, IL-1α, IL-1β, IL-6, TNF-α, Csf2, and Csf3; (b) chemokines Ccl2 and Cxcl10; (c) cyclooxygenase 2 (COX-2); (d) transcription factor NF-κB1; and (e) IκB-α. Whereas genistein shows a strong inhibition of the expression of these genes. Moreover, genistein treatment in LPS-stimulated macrophages could up-regulate the transcription factor IκB-β and c-Rel.

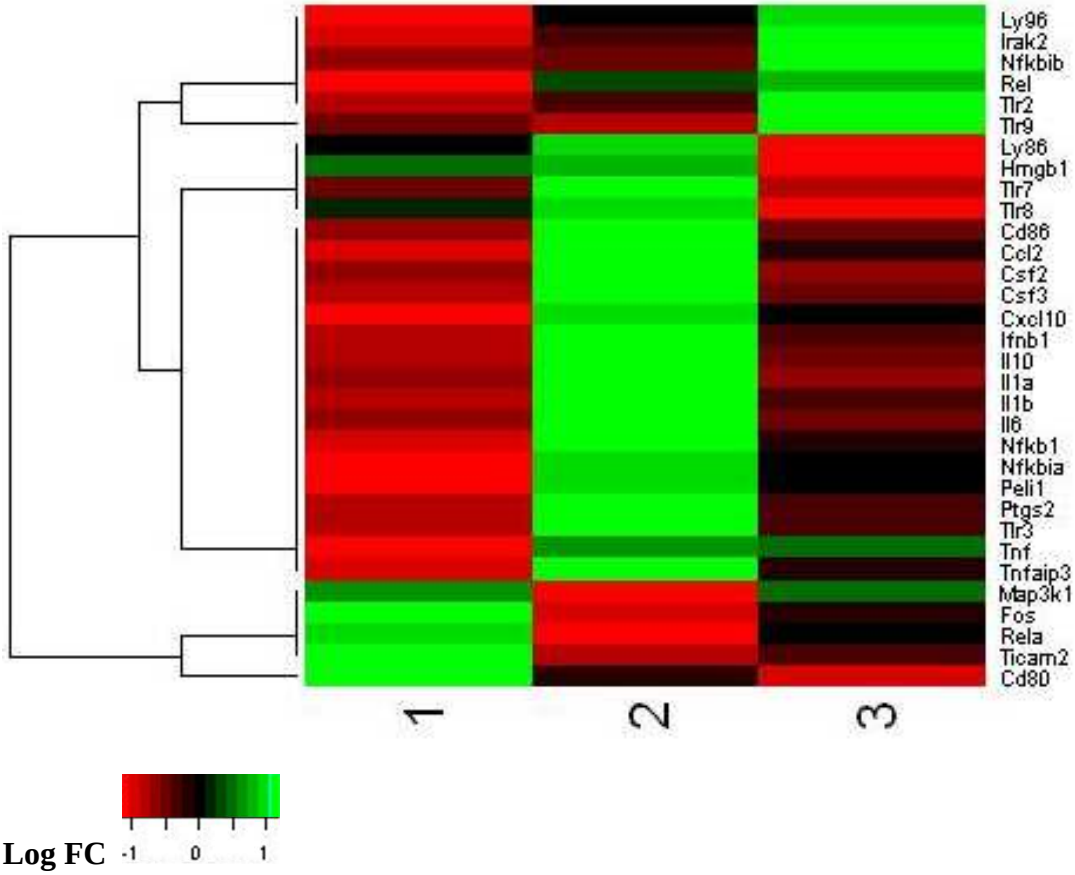


Fig. 4.11 Hierarchical clustering of 32 induced or repressed genes. Genes passing appropriate filter criteria were subjected to a hierarchical clustering. The homology tree plot is shown on the left side. 1: Genistein-treated (50μM) macrophages RAW 264.7; 2: LPS -treated (100 ng/ml) macrophages RAW 264.7; 3 means LPS+ Genistein treated macrophages RAW 264.7.

4.2 Effects of genistein on C. albicans

4.2.1 Genistein effects on the oxygen consumption of C. albicans and S. cerevisiae

In order to investigate the action of genistein on the mitochondrial function of *C. albicans* and *S. cerevisiae*, the rates of oxygen consumption in the presence of genistein (123-1.5 μM) were determined. For *C. albicans*, an inhibitory effect of genistein on oxygen consumption was observed (Fig. 4.12 A) after 10 min. Within the applied genistein concentration range from 1.5 to 123 μM, an influence of genistein concentration was observed. All cultures containing genistein showed higher oxygen concentrations, i.e. the respiratory rate was lower, than in cultures without genistein. However, for *S. cerevisiae*, no significant effect of genistein on oxygen consumption could be detected, even at the highest concentration of 123μM (Fig. 4.12).

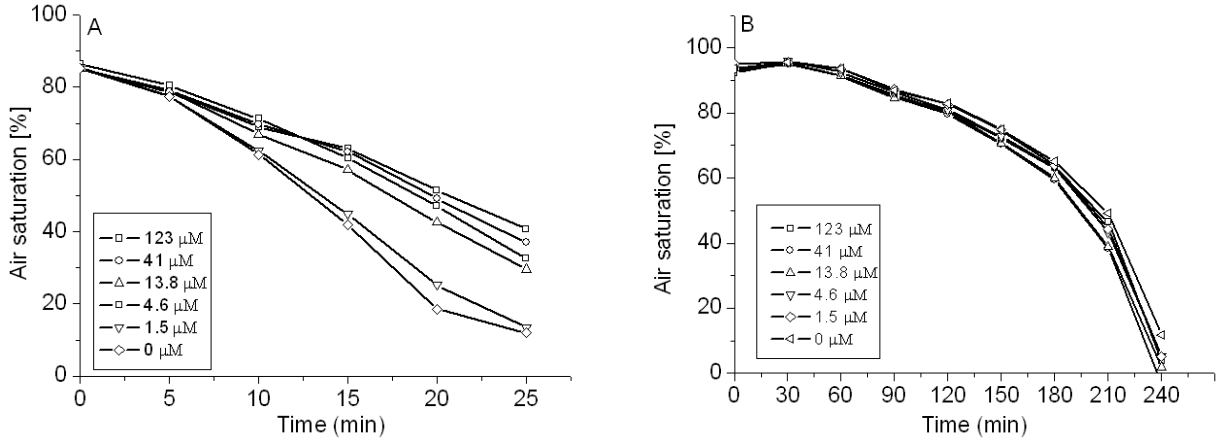


Fig. 4.12 Effect of genistein on the oxygen consumption of *C. albicans* (A) *and S. cerevisiae* (B). Oxygen consumption was measured in the OxoPlates® at the indicated times. The results represent two independent experiments.

4.2.2 Effect of genistein on the ROS accumulation in C. albicans and S. cerevisiae

Interference with the electron transport chain could lead to incomplete reduction of electron carriers, such as ubiquinones, which can induce the formation of reactive oxygen species (ROS) due to the subsequently incomplete reduction of oxygen. The fluorescent probe H_2DCFDA, is suitable for measurement of ROS formation in yeast cells [163], and was used to monitor ROS formation in *C. albicans* and *S. cerevisiae.* Genistein caused an increase in the fluorescence

intensity of *C. albicans* as shown in Fig. 4.13. After 2 h of incubation with genistein, the increased ROS generation was seen. At a concentration of 41μM, 13.8μM, and 4.6μM of genistein the increase was 1.26, 1.41, and 1.26 times compared with the control ($p<0.01$), respectively. These data indicated that genistein induced ROS accumulation in *C. albicans.*

On the other hand, genistein had no influence on ROS production of *S. cerevisiae* as shown in Fig. 4.13. After 2 h of incubation with genistein, no increase of ROS generation was observed. These data indicated that genistein did not induce ROS accumulation in *S. cerevisiae.*

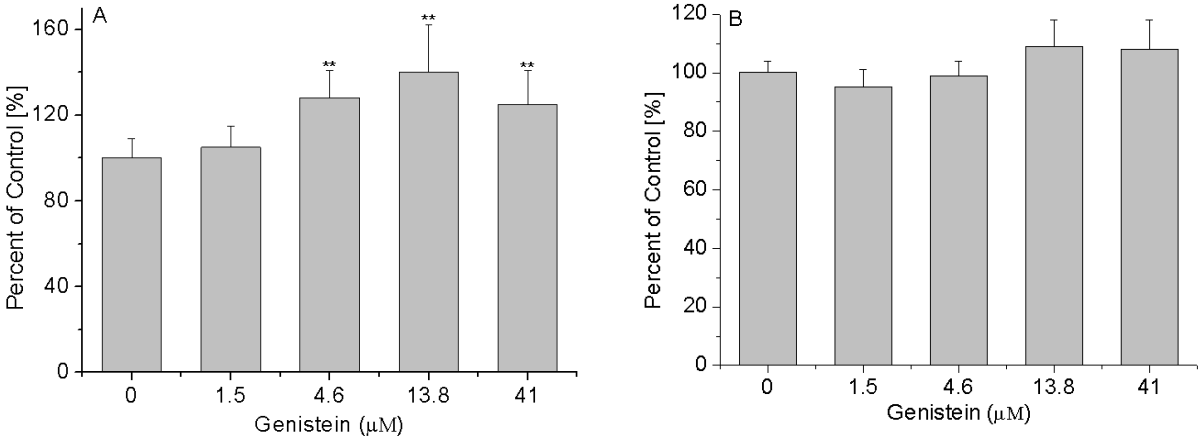


Fig. 4.13 Effects of genistein on the generation of endogenous ROS in *C. albicans* (A) and *S. cerevisiae* (B). Data represents the percentage of solvent control.

4.2.3 Effect of specific respiratory chain inhibitors on oxygen consumption and ROS production in C. albicans

4.2.3.1 Effect of rotenone on the oxygen consumption of *C. albicans* and *S. cerevisiae*

In the respiratory chain of *S. cerevisiae,* the classical complex I for NADH oxidation is missing [133]. Instead, it has two groups of rotenone-insensitive NADH dehydrogenases called NDI and NDE, which are NADH dehydrogenases placed at the inner (NDI) and at the external (NDE) side of the mitochondrial membrane. As the respiratory chain of *C. albicans* contains complex I, we speculated that this part of the respiratory chain may be the site of action of genistein. Thus, we used the specific complex I inhibitor rotenone to test its effects on oxygen consumption.

The rates of oxygen consumption in the presence of rotenone (34.8-3.9 μM) were determined. For *C. albicans,* a slightly inhibitory effect of rotenone on oxygen consumption was observed

(Fig. 4.14 A). However, for *S. cerevisiae*, no effect of rotenone on oxygen consumption was observed (Fig. 4.14 B).

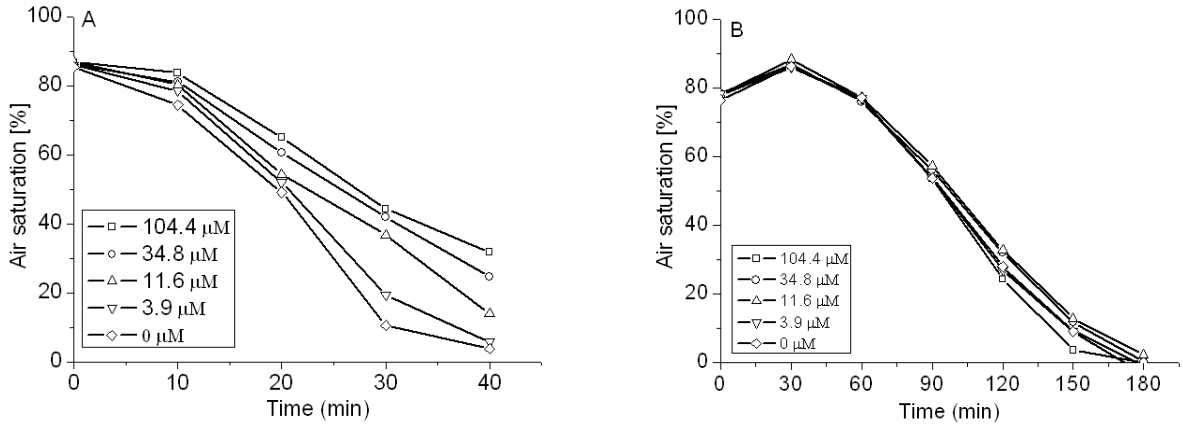


Fig. 4.14 Effects of rotenone on the oxygen consumption of *C. albicans* and *S. cerevisiae*. Oxygen consumption was measured in the OxoPlates® at the indicated times (A) *C. albicans* (B*) S. cerevisiae*

4.2.3.2 Effect of rotenone on the ROS accumulation in *C. albicans* and *S. cerevisiae*

Rotenone also caused an increase in the fluorescence intensity of *C. albicans* as shown in Fig. 4.15. After 2 h of incubation with rotenone, an increased ROS generation was seen for concentrations exceeding 10 µM. These data indicated that the specific complex I inhibitor rotenone induced ROS accumulation in *C. albicans* as well.

On the other hand, rotenone had no influence on ROS production of *S. cerevisiae* after 2 h of incubation with rotenone, suggesting that rotenone did not induce ROS accumulation in *S. cerevisiae.*

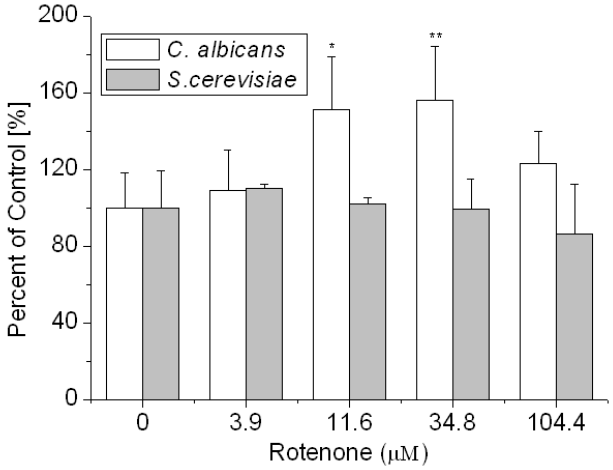


Fig. 4.15 Effects of rotenone on the generation of endogenous ROS in *C. albicans* and *S. cerevisiae.* Data represents the percentage of solvent control.

4.2.3.3 Effect of antimycin A on the oxygen consumption of *C. albicans* and *S. cerevisiae*

Antimycin A is a specific inhibitor of the complex III of the respiratory chain for both *C. albicans* and *S. cerevisiae*. The rates of oxygen consumption in the presence of antimycin A (16.4-0.6 μM) were determined. For *C. albicans*, a strong inhibitory effect of antimycin A on the oxygen consumption was already observed (Fig. 4.16 A) after 10 min. Moreover, for *S. cerevisiae*, antimycin A also showed a striking inhibition of oxygen consumption in the concentration range 16.4-0.6 μM (Fig. 4.16 B).

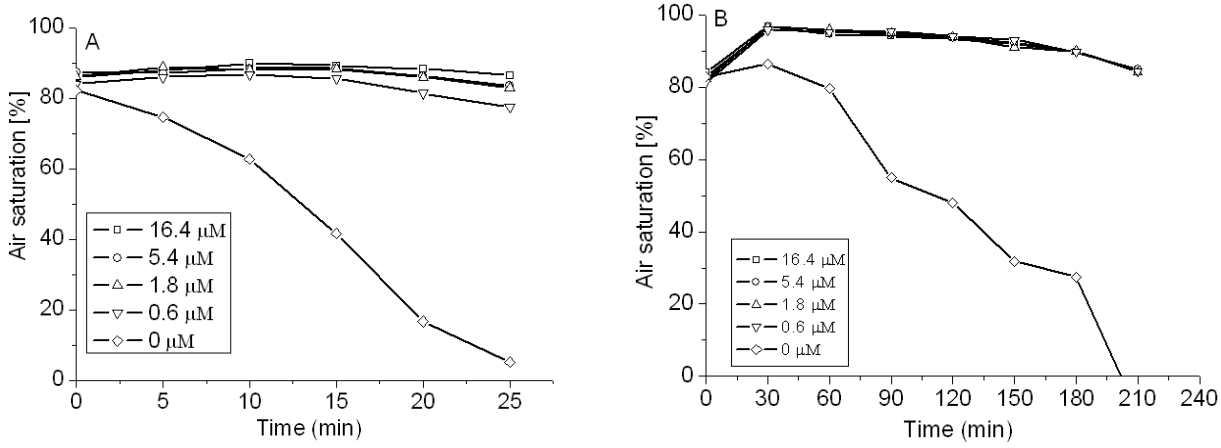


Fig. 4.16 Effects of antimycin A on on the oxygen consumption of *C. albicans* and *S. cerevisiae*. Oxygen consumption was measured in the OxoPlates® at the indicated times (A) *C. albicans* (B) *S. cerevisiae*).

4.2.3.4 Effect of antimycin A on the ROS accumulation in *C. albicans* and *S. cerevisiae*

Both *C. albicans* and *S. cerevisiae* showed a significant increase in ROS production when treated with antimycin A, as shown in Fig. 4.17. For *C. albicans*, at a concentration of 16.4 μM, 5.4 μM, 1.8 μM, and 0.6 μM of antimycin A, the increase was 2.05, 1.93, 1.84, and 1.85 times respectively compared with the control ($p<0.01$). On the other hand, antimycin A had a strong influence on ROS production of *S. cerevisiae* as it is shown in Fig. 4.17 B, which shows an increase of 1.68, 1.64, 1.62, and 1.60 times respectively compared with the control ($p<0.01$).

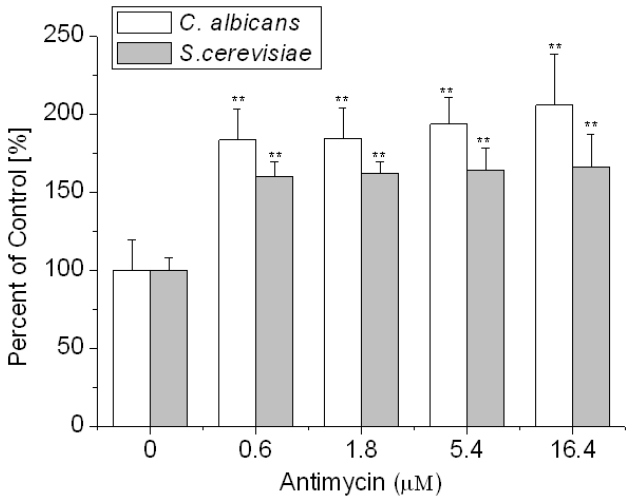


Fig. 4.17 Effects of antimycin A on the generation of endogenous ROS in *C. albicans* and *S. cerevisiae*. Data represent the percentage compared to solvent control.

4.2.4 Complex I activity

Dichlorophenolindophenol (DCIP) is used as artificial electron acceptor for complex I reductase activity. The oxidized form of blue DCIP can be reduced and turned into a colorless reduced form in the presence of *C. albicans* cells. Rotenone is an inhibitor of complex I, and thus inhibits the catalysed oxidation of NADH by DCIP. Fig. 4.18 shows DCIP reduction by the suspension of *C. albicans*. There is no difference between genistein-treated *C. albicans* and the control. However, rotenone strongly blocked the DCIP reduction. Thus, genistein shows a behavior different from rotenone.

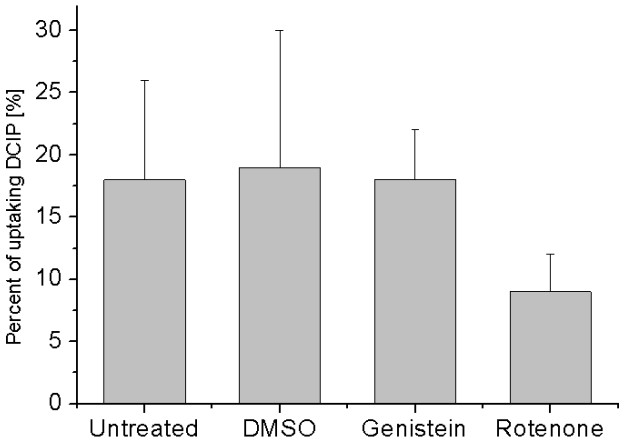


Fig. 4.18 The consuming of the DCIP color by suspensions cells of genistein (123 μM) and rotenone (63.3 μM) treated *C. albicans* after 20 min.

4.2.5 Ethanol production

As inhibition of the respiratory chain results in the redirection of metabolic pathways in favour of fermentative reactions, we detect ethanol in the culture supernatants (Fig. 4.19). The results showed that genistein (100 μM) led to a significant increase in ethanol productivities after treatment of *C. albicans* for 4 hours.

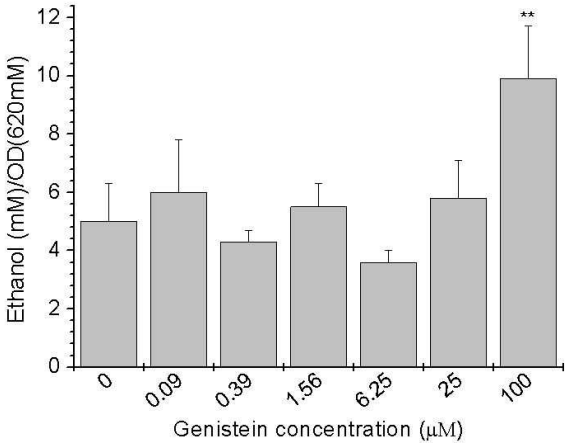


Fig. 4.19 Effects of genistein on the ethanol production of *C. albicans* after genistein treatment for 4 hours. The results represent at least two independent experiments.

4.2.6 Growth of C. albicans

Rotenone is a specific complex I inhibitor, which does not prevent *C. albicans* proliferation [198]. According to the literature, we tested whether genistein could influence the growth of *C. albicans*. Growth was followed via the determination of the turbidity at OD 620nm. As shown in Fig. 4.20, genistein (123 μM) had no effect on the growth rate of *C. albicans* from 0 - 5 hour

growth compared with DMSO control.

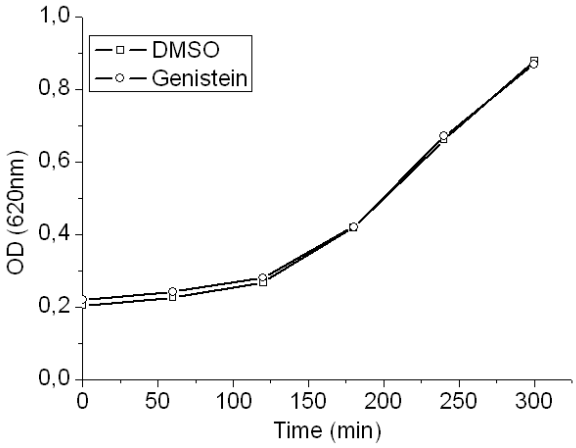


Fig. 4.20 Effect of genistein on growth of *C. albicans* in YPgal medium. The results represent at least two independent experiments.

4.2.7 Cell wall β-glucans and mannans components of C. albicans

β-1,3-glucans are reported to be essential for the recognition of *C. albicans* by the macrophage receptor dectin-1, the main receptor for phagocytosis of *C. albicans.* Therefore, we investigated whether genistein changed the cell wall structure and enhanced the accessibility of β-1,3-glucans on the surface of *C. albicans*. As shown in Fig. 4.21, treatment of *C. albicans* with genistein (100 μM) did not lead to an increase of the accessibility of β-1,3-glucans compared with DMSO control.

Mannan is another important cell wall constituent of *C. albicans*, which plays a role in the interaction between pathogen and host. So, we investigated whether genistein could influence the accessibility of mannans on the surface of *C. albicans*. As shown in Fig. 4.21, treatment of *C. albicans* with genistein (100 μM) did not lead to any influence of the accessibility of mannan compared with DMSO control.

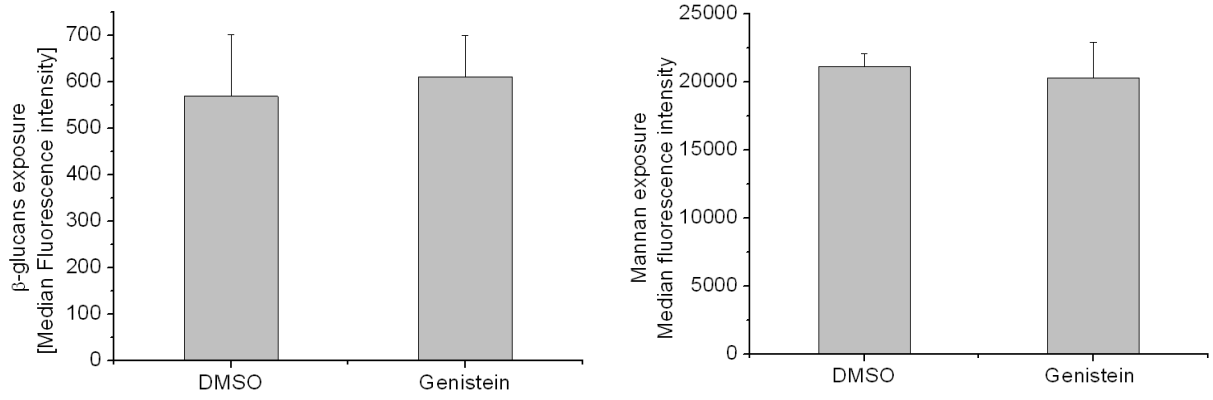


Fig. 4.21 FACS analysis of accessible β-1,3-glucans and mannan of genistein 100 μM treated *C. albicans* CAF2-1. The results presented are representative of three independent experiments.

4.2.8 Cell surface hydrophobicity of C. albicans

Cell surface hydrophobicity (CSH) was associated with an increased virulence of *C. albicans* due to stronger adherence to substrates [164, 165]. Therefore, we investigated the influence of genistein on the CSH of *C. albicans*. Hydrophobic and hydrophilic cells were separated using the biphasic separation method. As shown in Fig. 4.22, genistein (100 μM) treatment led to a 5 % increase of the cell surface hydrophobicity of *C. albicans* compared with the solvent control ($p<0.01$).

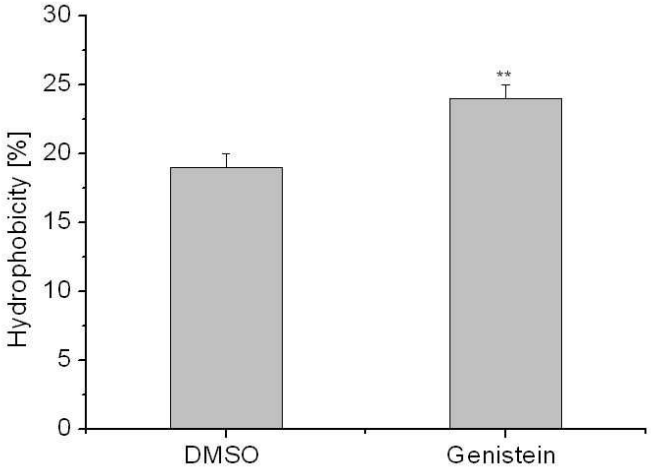


Fig. 4.22 Effect of genistein on the cell surface hydrophobicity of *C. albicans*. Results presented are representative of three independent experiments.

4.3 Infection

4.3.1 Receptor expression on macrophage surface related to C. albicans infections

Macrophages express high levels of Toll-like receptors (TLRs) on their cell membranes, as well as lectin receptors. Among them, Toll-like receptor 2 and dectin-1 receptor are important for recognition of *C. albicans* infections. The expression of the receptors may differ with different macrophage cell lines. Therefore, it is necessary to confirm the presence of receptors in the macrophage cell line RAW264.7. As shown in Fig. 4.23, macrophages RAW264.7 expressed high levels of TLR-2 and dectin-1 compared with the control which was only stained with the secondary antibody.

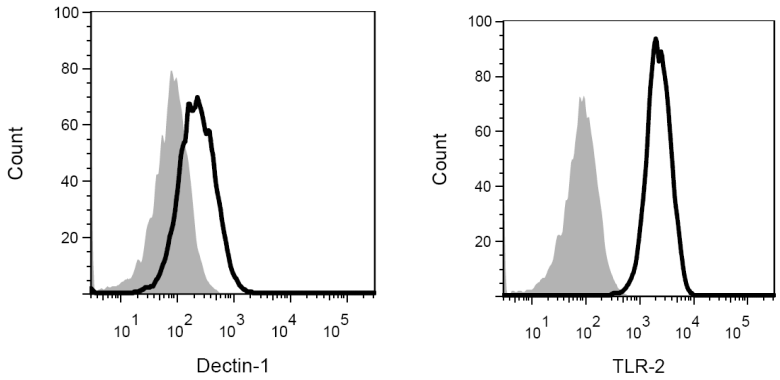


Fig. 4.23 Receptors expression of macrophage RAW264.7 by flow cytometry. The overlay of control: staining with only secondary antibody (grey area) and the first antibody staining (white area).

4.3.2 Phagocytosis of C. albicans

Genistein was reported to be a tyrosine kinase inhibitor with a rather broad specificity so that a number of signal transduction cascades could be influenced. Interactions of the pathogen *C. albicans* with host cells frequently lead to internalization of the yeast by processes such as endocytosis and phagocytosis. Endocytosis is of major relevance for non-phagocytotic host cells, such as vascular endothelial cells; whereas phagocytosis is an important function of macrophages. Both processes involve the phosphorylation of tyrosine residues and it was reported that endocytosis of *C. albicans* was significantly reduced by pretreatment of endothelial cells with genistein (100 µM) [32]. As phosphorylation of tyrosine residues are also downstream reactions of pathogen recognition by phagocytosis receptors [166], we investigated the influence of genistein on the phagocytosis of *C. albicans* by macrophages. As shown in Fig. 4.24, treatment of the macrophages with genistein reduced the phagocytosis efficiency in a time- and

dose-dependent manner. Pre-treatment of the macrophages with genistein for 2-8 hours did not influence the phagocytotic effect. However, when the pre-incubation time was extended to 24 hours, phagocytosis of *C. albicans* was decreased to 50% of the control by 25 µM genistein; and to about 80 - 90 % by 12.5 µM genistein, with a slight recovery after prolonged phagocytosis times (60 min). After 48 hours, the effects were even more pronounced, as the higher concentration (25 µM) led to the strong inhibition to 40%, and the lower concentration (12.5 µM) to less than 70%. The inhibition effect was observed throughout the whole observation period.

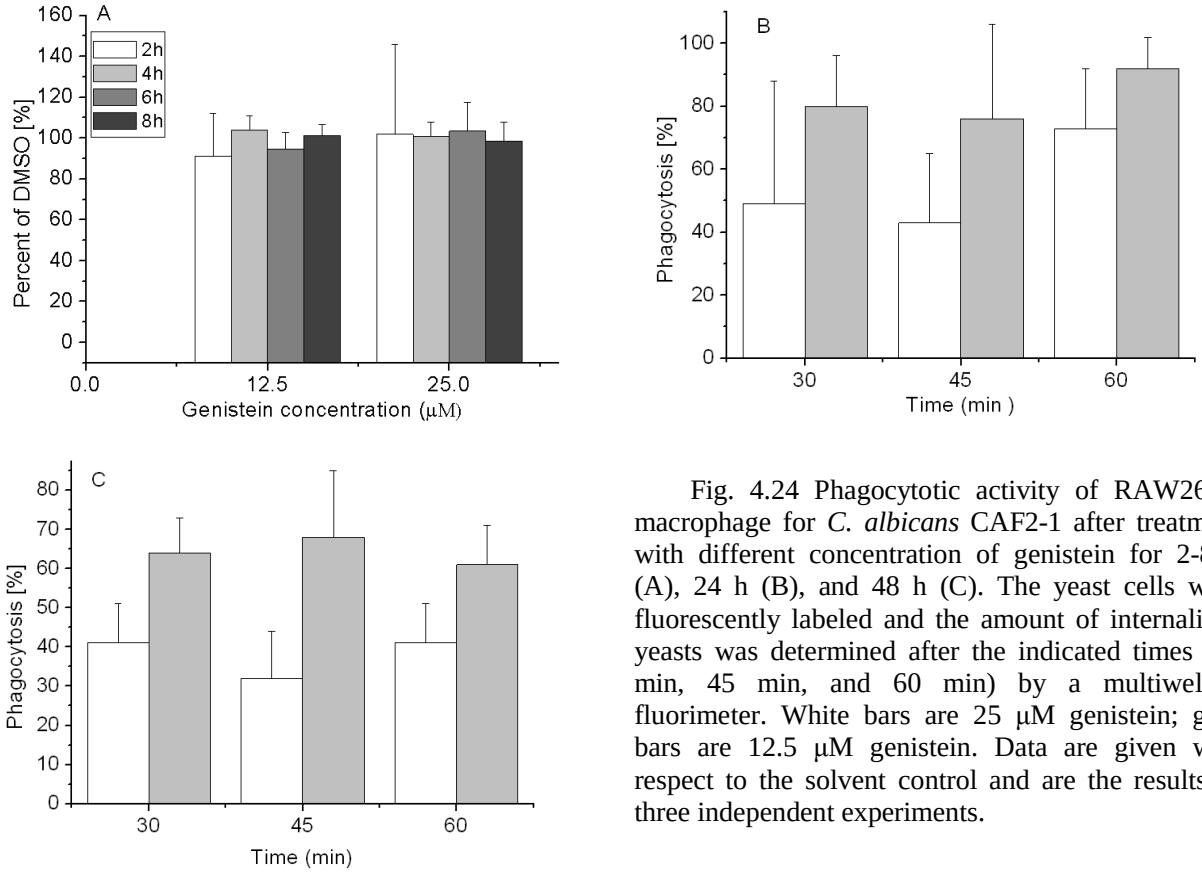


Fig. 4.24 Phagocytotic activity of RAW264.7 macrophage for *C. albicans* CAF2-1 after treatment with different concentration of genistein for 2-8 h (A), 24 h (B), and 48 h (C). The yeast cells were fluorescently labeled and the amount of internalized yeasts was determined after the indicated times (30 min, 45 min, and 60 min) by a multiwell - fluorimeter. White bars are 25 µM genistein; grey bars are 12.5 µM genistein. Data are given with respect to the solvent control and are the results of three independent experiments.

4.3.3 Cytokine production of genistein treated macrophages with C. albicans

We had observed that genistein reduced the cytokine production of LPS stimulated macrophages (4.1.7). As LPS mainly activates signal transduction cascades downstream of TLR4, whereas *C. albicans* also interacts with TLR2 and dectin-1, the influence of genistein on cytokine production by macrophages, which were stimulated with *C. albicans*, was also tested.

Macrophages were treated with genistein 1 hour before *C. albicans* was added. The supernatant was discarded after genistein treatment and the yeast suspension without genistein supplement was added, so that genistein could not influence *C. albicans.* As shown in Fig. 4.25, genistein led to a pronounced inhibition of TNF-α and IL-10 production after 1.5 and 5 hours infection times.

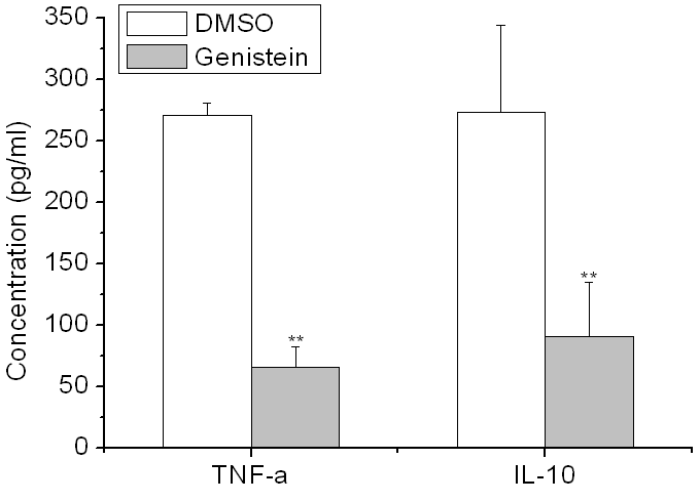


Fig. 4.25 TNF-α and IL-10 production of macrophages in response to *C. albicans* CAF2-1, influenced by genistein. The results represent two independent experiments, and data are the mean ± S.D. of at least 4 single values of each experiment.
**: $p<0.01$ compared with DMSO control.

4.3.4 Interaction of genistein-treated C. albicans with RAW264.7 macrophage

Genistein influenced the respiratory activity of *C. albicans*, the endogenous ROS accumulation, and cell surface hydrophobicity (4.2). As cell surface hydrophobicity may influence also the interactions to host cells, we tested whether genistein treatment of *C. albicans* could influence the degree of internalization by macrophages. The effect of genistein treatment of *C. albicans* on the time course of phagocytosis is shown in Fig. 4.26. Already at the earliest time point of 15 minutes incubation time of *C. albicans* with macrophages, the level of phagocytosis was significantly enhanced and this effect lasted during the whole experimental period of 60 minutes.

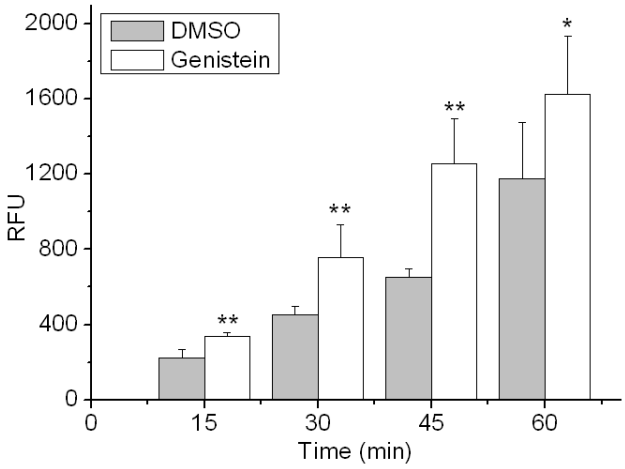


Fig. 4.26 . Phagocytosis of genistein treated *C. albicans* CAF2-1 by the macrophage cell line RAW 264.7. Data represent the mean ± S.D. for at least 6 single values in each experiment, each experiment was repeated 3 times. **: $p<0.01$; *: $p<0.05$ comparison with DMSO control.

4.3.5 Cytokine production of RAW264.7 macrophages stimulated with genistein-treated C. albicans

As we had observed an enhanced phagocytosis of genistein-treated *C. albicans*, we evaluated whether genistein treatment also influenced cytokine production. In Fig. 4.27, data are shown for the production of TNF-α and IL-10. Contrary to the results obtained, when macrophages were treated with genistein, the pre-incubation of *C. albicans* with genistein led to a significant enhancement of TNF-α concentrations and also to an increase of IL-10 concentrations ($p<0.01$).

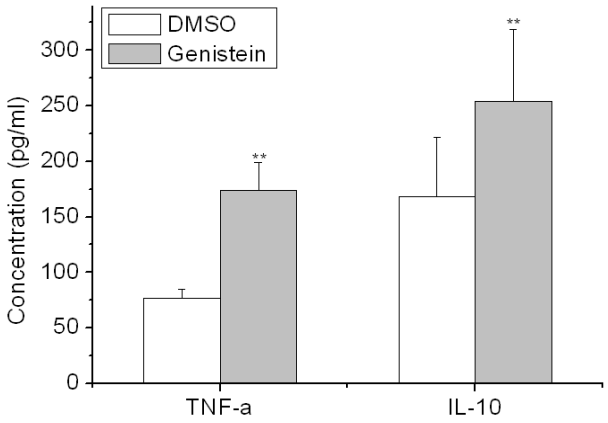


Fig. 4.27 Effects of genistein treatment of *C. albicans* CAF2-1 on the production of TNF-α (A) and IL-10 (B) by macrophages; the results represent two independent experiments, the data for each experiment are the mean of at least 4 single values of each experiment. **: $p<0.01$ comparison with DMSO control.

4.4 Interaction of C. albicans single gene deletion mutants with macrophages

4.4.1 Phagocytosis

As we had observed that treatment of *C. albicans* with genistein influenced the interaction to RAW264.7 macrophages, we used single gene deletion mutants of *C. albicans* to identify fungal pathways which are involved in this interaction. MAP kinase pathways and the histidine kinase Chk1p are involved in the cell wall structure of *C. albicans* [167, 168]. Therefore, we investigate whether there is any relation between MAP kinase pathways and histidine kinases, which may be the sensor proteins of the MAP kinases cascades. The MAP kinase mutants (*Δcek1*, *Δmkc1*, and *Δhog1*) and MAPK kinase mutants (*Δhst7* and *Δpbs2*) were used together with the histidine kinase mutant *Δchk1* and the reference strain CAF2-1 in the phagocytosis assay.

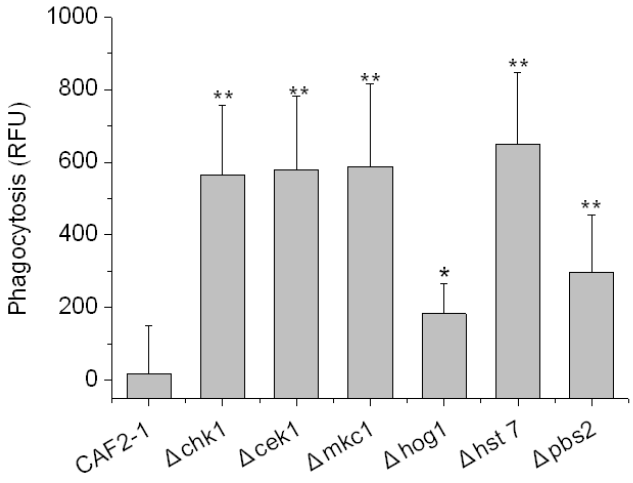


Fig. 4.28 Phagocytosis of MAP kinase mutants (*Δcek1*, *Δmkc1*, and *Δhog1*) and MAPK kinase mutants (*Δhst7* and *Δpbs2*) by RAW264.7 macrophages in comparison to the reference strain CAF2-1 and histidine kinase mutant *Δchk1*. The phagocytosis time was one hour. The figure shown above is the mean ± standard deviation of three independent experiments. **: $p<0.01$; *: $p<0.05$ comparison with CAF2-1.

Among the mutants, the MAP kinase mutant *Δhog1* showed only a small increase in phagocytosis efficiency compared with the reference strain (CAF 2-1). The mutant *Δpbs2* showed another slight increase which was to be regarded as significant. In contrast, the other MAP kinase mutants, *Δcek1* and *Δmkc1*, and the respective MAPK kinase mutant *Δhst7* were internalized significantly better by the RAW264.7 cells than the reference strain CAF2-1 (Fig. 4.28). Thus, effects from these strains were investigated further.

4.4.2 Cell wall β-glucans and mannans exposure of C. albicans mutants

β-1,3-glucans are reported to be essential for the recognition of *C. albicans* by the macrophage receptor dectin-1, the main receptor for phagocytosis of *C. albicans*. A previous study had shown that cell wall mannans of *CHK1*-deficient *C. albicans* were significantly truncated [169]. As in *C. albicans* the inner β-glucan layer of the fungal cell wall is known to be shielded by these mannan chains [54, 170], these shorter mannans could lead to an increased exposure of the β-glucans for immune recognition. Thus, the accessibility of β-1,3-glucans and mannans of MAPK and MAPK kinase mutants was evaluated in comparison to Δ*chk1* and the parental strain CAF2-1. Antibody-staining of the β-1,3 -glucans of CAF2-1 resulted in only poor fluorescence intensities of about 500 RFU (Fig. 4.30), whereas the MAPK and MAPK kinase

mutants as well as *Δchk1* were efficiently stained and achieved high median fluorescence intensities indicating a high amount of accessible β-1,3-glucans compared with CAF2-1 (Fig. 4.29). In addition, it has to be noted that background fluorescence observed from negative controls was similar for all mutants.

The cell wall mannans were stained with FITC-labeled concanavalin A and analysed by flow cytometry. The exposure of mannans of *Δchk1* was reduced to 60% of the level of CAF2-1. The decrease observed with *Δhog1* was not significant. *Δpbs2* still had 75% of the signal of CAF2-1, whereas all the other MAPK and MAPK kinase mutants showed mannan exposure levels of 60-70% of CAF2-1.

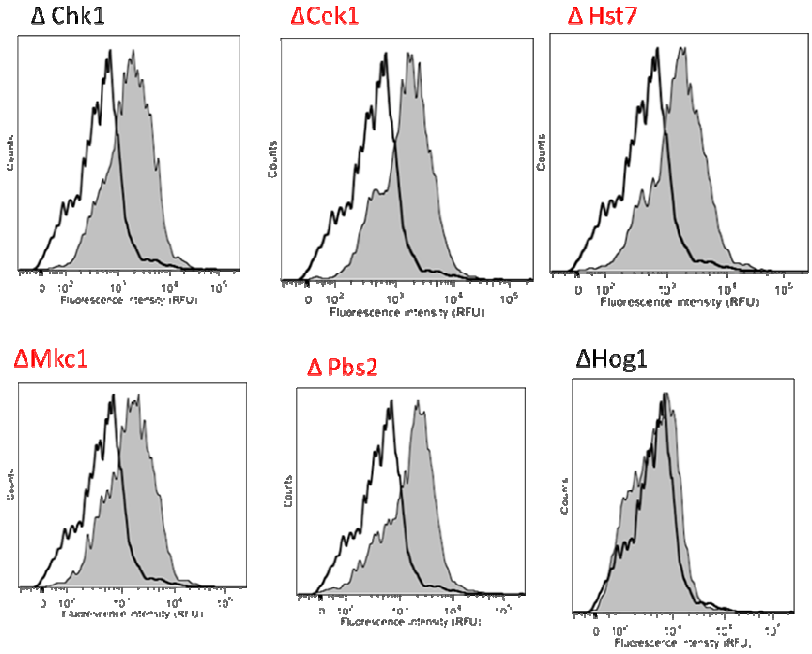


Fig. 4.29 Overlay of β-glucans expression of MAPK and MAPK kinase deletion mutants in comparison to *C. albicans* CAF2-1. Fluorescence intensity of antibody-labeled yeasts was detected via flow cytometry. The overlay of CAF2-1 (white area) and the respective mutants is shown (grey area). The mutants which have a higher exposure of glucans are highlighted in red.

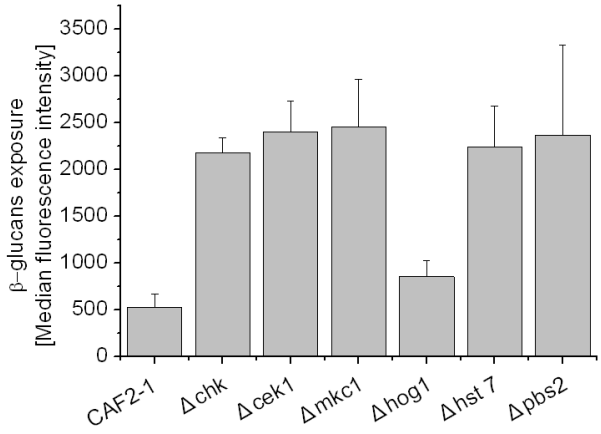


Fig. 4.30 Quantitative comparison of the median fluorescence intensities of stained mutants and the reference strain CAF2-1. Representative data of three independent experiments are shown.

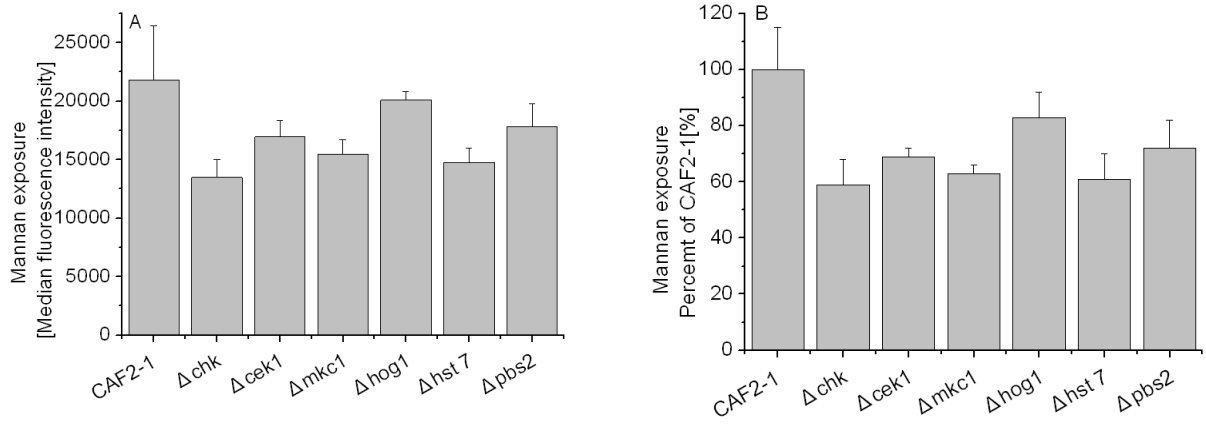


Fig. 4.31 Mannan exposure of MAPK and MAPK kinase deletion mutants in comparison to CAF2-1 and the histdine kinase mutant *Δchk*. (A) Yeast cells of the *C. albicans* mutant strains were stained with FITC labelled Concanavalin A. Fluorescence intensity of labeled yeasts was detected via flow cytometry. Quantitative comparison of the median fluorescence intensities of stained mutants and the reference strain CAF2-1. (B) Percentage of median fluorescence intensities of CAF2-1. Representative data of three independent experiments are shown.

4.4.3 Phagocytosis of C. albicans mutants by RAW264.7 macrophage after genistein treatment

MAP kinase pathways are used by eukaryotic cells to response to changes in the external environment. Although there are several differences between mammalian cells and lower eukaryotic cells regarding the complexity and functionality of the elements involved, the core structure of the MAP kinase cascade is highly conserved [171]. As genistein could activate the

MAPK kinase signalling cascade by phosphorylation of MAP kinase p38 and ERK1/2 in mammalian cells, we utilized genistein-treated MAP kinase deletion mutants with genistein to detect whether genistein could target on the MAPK kinase pathways in *C. albicans*. We analyzed the phagocytic efficiency of macrophages for genistein treated *C. albicans* mutants. The result showed that the phagocytosis efficiency for *Δcek1* and *Δhog1* was not affected by genistein treatment, suggesting that the genistein effect of the wild type could be related to the pathways.

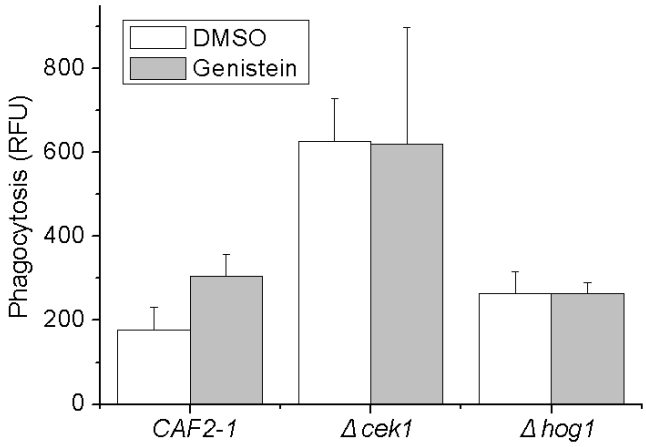


Fig. 4.32 Phagocytosis of genistein treated mutants by RAW264.7 macrophages in comparison to the reference strain treated CAF2-1. The phagocytosis time was 45 minutes. The figure shown above is the mean ± standard deviation of 4 repeated values.

5 DISCUSSION

5.1 Effects of genistein on the macrophages

Genistein, an isoflavone, has been shown to inhibit the proliferation of several types of cancer cells. However, currently little is known about its effect on macrophages. Our experiments focused on the effects of genistein on the murine macrophage cell line RAW264.7. Different concentrations of genistein were used to examine the effects of genistein on macrophage proliferation, cell cycle, cell morphology and F-actin cytoskeleton structure.

In this study, the WST-1 method was used to detect cell proliferation. The WST-1 method is easier and more sensitive compared with the conventional MTT method. In metabolically active cells, the colorless tetrazolium salt is reduced by dehydrogenases to a yellow water-soluble formazan. This color change can be detected by a spectrophotometer at the wavelength of 450 nm. The results showed that genistein had an inhibitory effect on the cell viability in a time- and dose-dependent manner (4.1.1). When the macrophages were treated with genistein for the short time of only 2 h, none of the genistein concentrations led to decreased signals. However, when the incubation time was extended to 24 and 48 hours, genistein concentrations in the range of 50-100μM reduced the cell viability. But no influence on the cell viability was observed with lower genistein concentrations (12.5, 25 μM). Meanwhile, genistein arrested the macrophages at the G2/M phase of the cell cycle in a concentration- and time-dependent manner. When the macrophages were treated with genistein for 24 hours, the number of cells in the G2/M phase significantly increased when the concentration was 100 or 50 μM, respectively. The effect was more striking when the incubation time was extended to 48 hours. Lower concentrations (25 and 12.5 μM) of genistein had no obvious effect on the cell cycle, suggesting that the reduction of cell viability was due to reduced cell proliferation, which was due to cell cycle arrest (4.1.2).

Genistein treatment also altered the morphology of RAW264.7 cells via influencing the actin cytoskeleton structure (4.1.3-4.1.4). An inverted contrast microscope was used to observe the cells treated with genistein for 2, 24, and 48 hours. Aberrations in cellular morphology were observed and the morphological changes were time- and dose-dependent. The different concentrations of genistein exerted no effects on the morphology after 2 hours of treatment. After 24h, genistein-treated cells were well attached, but the cell size increased and showed irregular shapes such as diamond shapes or pseudopodia-like protrusions. After 48h, these morphological

changes became even more obvious. The morphology change is consistent with the results from cell proliferation and cell cycle.

As the morphology of cells is at least partly determined by the cytoskeleton, we investigated the structure of the actin filaments in cells, which were treated with genistein. We could clearly observe the elongated actin filaments leading to the above mentioned protrusions, and the spindle morphology with actin filaments at the edge of the cells. The effect of genistein occured after a longer treatment time than effects of cytochalasin B (an actin polymerization inhibitor), which exerts its effect already after 2 hours [152]. Therefore, we speculated that the genistein effect could be a secondary effect, such as the result of inhibition of different tyrosine kinases in actin signal transduction pathways, which subsequently may result in changes in cytoskeleton dynamics. The results also showed that genistein caused the changes via different mechanism than the cytoskeleton inhibitor cytochalasin B. The results laid a basis for further investigations, especially on the phagocytic function of macrophages for *C. albicans.*

Genistein has been reported to suppress tumor cell growth, an effect which is mediated by different types of cell cycle arrest in the G2/M phase in the several tumor cell lines. Examples of such tumor cells include hepatoma cells, breast cancer cells, neoplastic human mammary epithelial cells, lung cancer cells, gastric cancer cells, and prostate cancer cells [156, 172-176]. Recent studies have shown that genistein can block the human epithelial cell line A549 cells in G 2/M phase, alter their morphology, and depolymerise interphase microtubules [177]. MAP kinase pathways were shown to be implicated in G2/M phase regulation and genistein may exert its influence on the cell cycle via activation and inhibition of distinct MAP kinase pathways [47-49]. Our results showed for the first time that genistein inhibited the proliferation of macrophages via arrests of cells in the G2/M phase in high concentrations. But whether genistein could activate the p-ERK1/2 and p-p38 of macrophage needs to be further investigated.

In conclusion, our comprehensive results clearly showed that only extended incubation of macropahges with genistein affected proliferation, cell cycle, cell morphology, and cytoskeleton structure. The results provided a basis for further studies of genistein on macrophage functions.

5.2 Genistein shows a strong anti-inflammatory effect on LPS-stimulated macrophages

Genistein has a variety of pharmacological effects, such as anti-cancer, anti-inflammatory, immune modulatory, and cardiovascular protection effects. Excessive secretions of inflammatory

factors can cause severe disease such as septic shock. Effective regulation of pro-inflammatory cytokine secretion has a potential clinical application as it will effectively control the inflammatory response, but could also lower the infectivity of some opportunistic pathogens such as *C. albicans* and *Aspergillus fumigatus.* However, the anti-inflammatory effects of genistein in particular on activated macrophages RAW264.7 had not been investigated in depth. Therefore, we studied effects of genistein on the response of macrophages to LPS, IFN-γ, and IFN-γ + LPS. A possible mechanism of genistein especially on LPS-activated macrophages is discussed.

LPS is a membrane component of gram-negative bacteria, acts as an endotoxin and elicits strong immune responses in animals. It activates macrophages via the Toll-like receptor 4 signal transduction pathway to activate nuclear transcription factors NF-κB and AP-1, leading to a release of inflammatory mediators like NO, TNF-α, IL-1β, IL-6, and IL-10. Thus, in the laboratory, LPS mimics the inflammation state in the body. Genistein could strongly inhibit LPS-induced NO and secretion of inflammatory cytokines TNF-α, IL-6, and IL-10 in the RAW264.7 macrophages in a dose-dependent manner. It has been reported that pre-treatment with genistein before intraperitoneal injection of LPS in a murine inflammation model significantly lowered the serum levels of TNF-α *in vivo* [178]. Moreover, genistein significantly inhibited cytokine-induced increases of TNF-α, interleukin-1β, IL-8 mRNA, and protein expression of inflammatory mediators in human brain microvascular endothelial cells [179]. Our results confirmed that genistein has a potential anti-inflammatory effect.

IFN-γ is a cytokine that is critical for innate and adaptive immunity against viral and intracellular bacterial infections as well as for tumor control. IFN-γ not only inhibits viral replication but also shows immunostimulatory and immunomodulatory effects [180]. Aberrant IFN-γ expression is associated with a number of autoinflammatory and autoimmune diseases. IFN-γ activates the Jak-STAT pathway in macrophages and induces production of NO and cytokines. Genistein strongly abrogated both IFN-γ-induced and LPS-induced release of NO in a similar way. The effect of the genistein on the IFN-γ pathway is currently not well understood. Our results showed for the first time that genistein also influences the IFN-γ-activated Jak2-STAT signalling transduction pathways.

Tyrosine phosphorylation is one of the earliest events to occur in response to an immune challenge, but the role of specific tyrosine kinases in inflammatory cytokine production has been difficult to ascribe due to conflicting literature. As genistein is a broad range tyrosine kinase inhibitor, we additionally applied specific tyrosine kinase inhibitors (Jak2, Syk, and Src inhibitors), MAP kinase inhibitors (SB203580, PD98059), inhibitors of down-stream kinases (PI3

kinase and Akt kinase inhibitors), and a protein phosphotyrosine phosphatase inhibitor (Bpv (phen)). We found that both tyrosine kinases Jak2 and Syk are essential for NO production as a response to LPS and IFN-γ activation. Until now, only Jak2 is known to be involved in the IFN-γ signaling pathway, our results now showed the decisive role of tyrosine kinase Syk. However, we did not find an essential role for the MAP kinase ERK and the protein phosphotyrosine phosphatase in both the LPS and the IFN-γ pathway, and p38 was required only for LPS stimulated NO production. The results do not correlate with another report, in which an regulating role of the ERK pathway in the IFN-γ-induced macrophage NO production and an enhancement by phosphatase inhibition was observed [181]. However, all kinases and phosphatases were involved in TNF-α, IL-6, and IL-10 production in a dose-dependent manner. The results suggest that the different kinases and the phosphatase functions in a dose-dependent manner. As tyrosine phosphorylation is one of the rapid responses to the stimulus, after a long time period stimulation like in the NO assay, the effect of some kinases could be compensated by the cross talk with other pathways. From these results, we concluded that the MAP kinases, ERK1/2, and p38 are involved in the short time response of LPS activation for cytokine production; whereas, tyrosine kinases are involved in both processes. This could explain the inhibitory effect of genistein, a broad spectrum tyrosine kinase inhibitor, on NO and cytokines production independent of the stimulus.

To further delineate and confirm the signalling processes involving MAP kinases in the regulation of LPS-induced activation of macrophages by genistein, we evaluated the induction of their phosphorylation by western blot analysis (4.1.9). LPS led to the phosphorylation of Raf, MEK1/2, and ERK1/2 already after 15 min post-stimulation and it sustained till 30 minutes. Phosphorylation of p38 was also observed, but it was not as obvious as p-ERK1/2. The results also proved that both p38 and p44/42 mitogen-activated protein kinases are involved in LPS activation of RAW264.7, which is also partly confirmed in the literature [162]. Addition of genistein further increased the phosphorylation of ERK1/2, of its downstream protein p90rsk and of p38. These results differed from the ones observed by Chu [182], who showed that LPS activation of MAPK was significantly depressed by genistein, SB 203580, and PD 98058 in the human monocytic THP-1 cells. The discrepancy between our results and those reported previously could be due to the different cell lines, the pre-treatment time for inhibitors, and the stimulation times for LPS. Meanwhile, genistein has been reported to selectively potentiate arsenic trioxide-induced apoptosis in human leukaemia cells via activation of p38 mitogen-activated protein kinase [48]. Our results were the first to suggest that genistein activated

p-p38 and p-ERK1/2 in LPS-stimulated macrophages, but the clear mechanism still requires further investigation.

At the transcriptional level, the NF-κB and AP-1 transcription factors play a key role in the regulation of inflammation and therefore, in the generation and release of NO and cytokines like TNF-α, IL-6, and IL-10. NF-κB plays important roles in the control of cell growth, differentiation, apoptosis, and stress-response. Whereas, NF-κB activation is part of the TLR4 signalling pathway, and the activation of AP-1 is related to the MAPK signal transduction pathway. To further elucidate the anti inflammatory role of genistein, we first used the RAW-Blue™ cells to detect the effect of genistein on both the nuclear transcription factor NF-κB and AP-1 (4.1.10). RAW-Blue™ cells could stably express the secreted embryonic alkaline phosphatase (SEAP) gene, which is induced by activation of either the NF-κB or the AP-1 transcription factor. The results showed that genistein significantly reduced the expression of the reporter protein and thus inhibited simultaneously NF-κB and AP-1 in a dose-dependent manner.

In order to study genistein effects on the macrophages at the transcriptional level, we used a Toll like receptor pathway PCR array to investigate expression of related genes after different treatments (4.1.11). Cells treated with (i) LPS and (ii) LPS together with genistein demonstrated significant number of genes to be differentially regulated compared to control cells. Genistein alone could up-regulate the gene CD80, MEKK1, c-fos, Rela, and Ticam2. LPS could up-regulate 20 genes including (a) cytokines IFN-β, IL-10, IL-1α, IL-1β, IL-6, TNF-α, Csf2, and Csf3; (b) chemokines Ccl2 and Cxcl10; (c) transcription factor NF-κB1; (d) IκB-α; and (e) cyclooxygenase 2 (COX-2). The presence of genistein led to a strong inhibition of the expression of these genes and upregulated the transcription factors IκB-β and *c-Rel*, a subunit of NF-κB. Genistein has been shown to inhibit the COX-2 production in LPS-stimulated chondrocytes [183]. The results correlate to the data obtained from IL-10, IL-6, and TNF-α protein secretion.

The results also showed that genistein alone could activate MEKK1 and *c-Fos* gene expressions. Members of the *Fos* family dimerise with *c-jun* to form the AP-1 transcription factor. In the previous results, we found genistein could inhibit the activation of transcription factor NF-κB and AP-1 in the RAW-Blue™ reporter cell line. This results confirmed the knowledge that genistein could inhibit the expression of NF-κB1 and IκB-α at the transcriptional level. Additionally, these results provide additional evidence for the results from western blot that genistein could activate MAP kinase cascade.

In conclusion, genistein showed a strong anti-inflammatory effect in RAW264.7 macrophages: it inhibited the secretion of NO and reduced the secretion of inflammatory

cytokines such as TNF-α, IL-6, and IL-10 via its effects on the transcription of these proteins. Moreover, genistein could strongly regulate the gene expression of cytokines, such as IL-1α, IL-1β, IFN-β, Csf-2, and Csf-3, and of chemokines, such as Ccl2 and Cxcl10. The effects of genistein could partly be due to tyrosine kinase inhibition of JAK2, Syk, Src and their downstream kinase PI3K-AKT pathway leading to the inhibition of the activation of the transcription factors NF-κB and AP-1. The results of this study provided more evidence for investigation of the mechanism of anti-inflammatory effects of genistein.

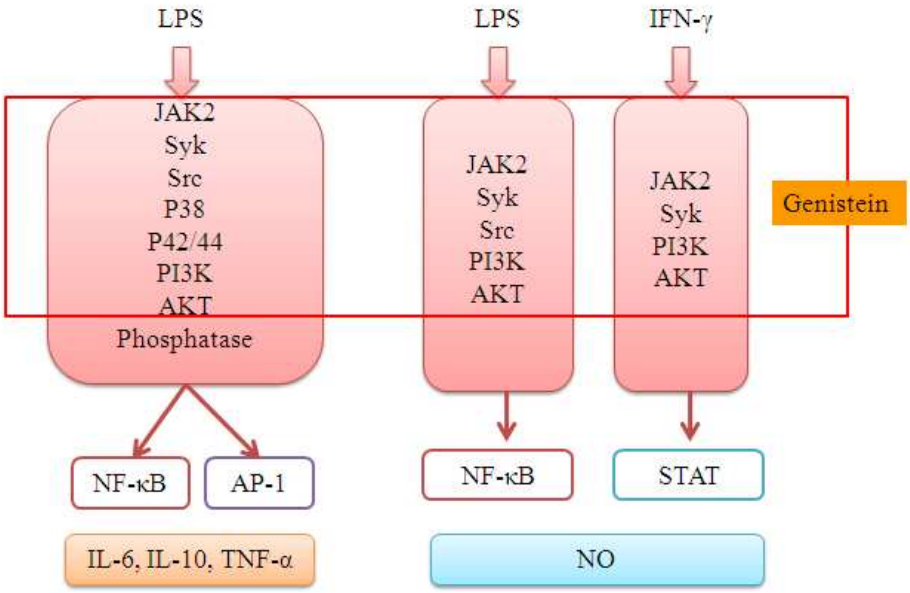


Fig. 5.1 Schematic illustration of the inflammation mechanism in macrophage RAW264.7 activated by LPS and/or IFN-γ. The kinases which are potentially targeted by genistein based on our results are highlighted by a red rectangle.

5.3 Genistein effects on C. albicans

Mitochondria are known as the power houses of the cell, they play very important roles in a range of processes such as ATP production, electron transport, and oxidative phosphorylation, release of caspase-activating proteins, generation of reactive oxygen species (ROS) and changes of cellular redox potentials [124]. Due to these properties, mitochondria are considered to be a potential target for antifungal agents. Compounds such as rotenone, antimycin, myxothiazols, melithiazols and cystothiazoles are known to exert their antifungal activity by specifically inhibiting the electron transport within the respiratory chain [125, 126]. In plants, flavonoids are also responsible for defense against infections. Flavonoids, such as hispidulin, resveratrol, eupafolin, genistein, and flavone, target on the respiratory chain of mammalian cells [184-188].

However, the function of flavonoids, on *C. albicans* is still unknown. Therefore, it is interesting to investigate genistein effects on *C. albicans* especially on mitochondria.

Genistein showed an inhibitory effect of oxygen consumption and induced ROS production in *C. albicans* but not in *S. cerevisiae*, suggesting electron transport chain could be the target of genistein. Rotenone, a specific complex I inhibitor, also showed an inhibitory effect of oxygen consumption and an increased ROS production only in *C. albicans*. *S. cerevisiae* lacks the classical complex I and thus is resistant to rotenone, whereas, *C. albicans* has NADH dehydrogenases and complex I.

Antimycin A is a specific complex III inhibitor, which strongly blocked oxygen consumption and significantly increased ROS production both in *C. albicans* and *S. cerevisiae*, suggesting that inhibition of complex III is prominent for oxygen consumption and ROS production for both *C. albicans* and *S. cerevisiae*. This result also indicated that genistein did not target on the complex III.

In contrast to *S. cerevisiae*, *C. albicans* expresses an alternative oxidase (AOX) that act downstream from coenzyme Q. It is induced by inhibitors of complex III and IV, such as antimycin A or cyanide [132]. This alternative oxidase is related to cyanide resistant respiration, which accepts electrons from the ubiquinone pool of the main cytochrome pathway and reduces oxygen to water [132]. There are also reports that reactive oxygen species (ROS), such as superoxide radical anions and H_2O_2 induce the expression of AOX in *Hansenula anomala* and *Petunia hybrid*, suggesting that the cyanide-resistant respiration may be part of defence system against oxidative stress, minimizing the generation of ROS in mitochondria [189, 190]. Since we observed an increase in the level of ROS, we excluded the possibility that genistein could target on the alternative oxidase. Thus, we assumed that complex I is the most likely target of genistein.

Complex I is a very large enzyme at the entry point of the mitochondrial electron transport chain [191, 192]. It has a complex structure and a number of redox centers involved in the electron transfer from NADH to a lipid soluble electron carrier, ubiquinone or Coenzyme Q (CoQ). The redox reactions are performed by one non-covalently bound FMN, at least six iron-sulfur clusters, and at least two distinct protein-bound species of quinone [193]. The FMN is considered to be the direct electron acceptor of NADH and subsequently electrons are transferred to the iron-sulphur clusters. N-2 iron-sulphur center is the direct electron donor to endogenous ubiquinone. Complex I is also considered to be one of the main sites of reactive oxygen species (ROS) production. Electrons leaked at complex I can reduce oxygen and give rise to the superoxide anion. N-2 iron-sulphur cluster was reported to be the site of the electron leak, but

N-2–ubisemiquinone region, ubisemiquinone, FMN and iron–sulphur cluster N1a have also been proposed as electron donors to oxygen [192].

As the mechanism of superoxide production by complex I is not yet clear due to a lack of knowledge about the exact sequence of the electron carriers and on the coupling of the electron transfer to proton translocation [192], the mechanism of action of genistein cannot be precisely defined. But we propose that genistein could act as a prooxidant by its direct reaction with the iron of complex I of the respiratory chain and O_2. Cao et al. have proposed a reaction sequence for ROS production where the first step requires the oxidized state of the involved transition metal, which is reduced by the reaction with genistein [194]. The generation of the hydrogen peroxide can be assumed by the following reaction sequence [186, 194].

$$2Fe^{3+} + \text{Gen-OH} \longrightarrow 2Fe^{2+} + \text{Gen}{=}O + H^+$$

$$2Fe^{2+} + O_2 \longleftrightarrow FeO_2^{2+}$$

$$FeO_2^{2+} + Fe^{2+} + 2H^+ \longrightarrow 2Fe^{3+} + H_2O_2$$

GEN-OH is the reduced form of genistein, GEN=O is the oxidized form and $Fe^{3+/2+}$ is part of an Fe-S cluster belonging to the involved respiratory chain complex.

We further tested whether genistein influenced the complex I reductase activity. We used DCIP as an electron mediator, DCIP is a hydrophilic electron acceptor widely used to test complex I reductase activity. It is reported that only 20-30% of this activity is sensitive to rotenone [192]. The results showed that genistein and rotenone affect DCIP reduction in a different way. Rotenone prevented this reduction, while genistein has no effect. This suggests genistein did not target the rotenone sensitive NADH dehydrogenase. Genistein was reported to inhibit the activity of complex I in bovine heart electron transport particles but was considered to be less active than rotenoid compounds [195].

As glucose was the carbon source in the cultivations, the main pathway for the carbon metabolism was glycolysis. In aerobic organism *C. albicans*, respiration is the main pathway for energy generation [146]. The glucose will convert to pyruvate together with the production of adenosine triphoshpate (ATP) and NADH. NADH oxidation in the respiratory chain leads to electron transfer from NADH finally to oxygen and production ATP. Inhibitors of respiratory chain redirect pathways to fermentation and thus enhance the ethanol and glycerol production [148]. Thus, inhibition of the respiratory chain by genistein should influence the ethanol production. We confirmed that genistein indeed slightly induced ethanol production at the highest

concentration of 100 μM.

In conclusion, our results showed that genistein targeted on the respiratory chain of *C. albicans*. We propose that genistein is oxidized by a redox reaction with iron-sulfur clusters and thus blocks electron transport and consequently oxygen consumption and induces ROS production. Our results also confirmed that growth rates of yeasts are not necessarily correlated to respiration rates. The parallel analysis of effects on growth rate and on the oxygen consumption rate improves the reliability of the detection of potential inhibitors and gives additional hints on the mode of action of compounds [146].

5.4 Treatment of C. albicans with genistein enhances the activity of murine macrophages

Anti-inflammatory properties of genistein have been investigated. Up to now, most experiments are focused on the pre-treatment of different cells of the immune system with genistein and on studying the resulting influences on the functions of the immune system. For example, macrophages [196-200], neutrophils [201-206] and monocytes [207] were treated with genistein prior to stimulation with LPS or infection by different pathogens such as *E. coli* [208], *Legionella pneumophila* [207], and *Cryptosporidium parvum* [209]. Usually inhibitory effects on the functions of the immune cells were observed, which could be explained mainly by the inhibition of tyrosine kinases by genistein. In our study, the effect of genistein on the interaction between *C. albicans* and macrophages was evaluated, particularly, the effects of genistein on phagocytosis of *C. albicans* by macrophages considering two different aspects: genistein acts either (i) on the host or (ii) on the pathogen. The results showed that genistein could act on both the yeast and the macrophages.

According to previous reports, tyrosine residues in host cell proteins became phosphorylated when host cells interact with *C. albicans* [31, 32]. As the endocytotic internalization of *C. albicans* by endothelial cells was one of the affected processes [37], we investigated whether incubation of macrophages with genistein also influenced internalization of *C. albicans* by these cells, which is mainly due to phagocytosis. We did not observe short-term effects, but extension of the incubation time to 24 or 48 hours significantly reduced the phagocytotic activity without influencing the cell viability [210]. This observation correlates to the changes in cell morphology and in the structure of the actin cytoskeleton, which were also observed only after 48 hours of incubation. Previously, it was shown that any interference with the dynamics of the actin

cytoskeleton leads to a reduction of the phagocytotic efficiency of macrophages for *C. albicans* [211]. Thus, the inhibition of phagocytosis after extended genistein incubation time may be due to secondary effects of genistein on the cytoskeleton.

However, inhibition of the secretion of the cytokines TNF-α and IL-10 occurred after a pre-incubation time of only 1 hour, which indicates direct effects on signal transduction cascades. Similar effects of genistein on TNF-α and IL-10 production were observed when macrophages were treated with LPS. Moreover, dectin-1 together with Toll-like receptor 2 (TLR2) are involved in cytokine production resulting from the presence of *C. albicans* [84], and the corresponding signalling cascades comprise of several tyrosine kinases. FACS analysis showed that dectin-1 and TLR2 receptors are expressed on the surface of RAW264.7 macrophages. The inhibitory effects of genistein on the production of these cytokines are probably due to the tyrosine kinase inhibitory property of genistein.

Further, we investigated whether treatment of *C. albicans* with genistein influenced the interaction to host cells. We cultivated *C. albicans* in the presence of genistein overnight to allow not only immediate effects, but also secondary effects such as the cell wall composition, which is the primary point of interaction to host cells. Contrary to the results obtained when macrophages were treated with genistein, we observed an enhanced level of phagocytosis and also a stimulated production of the cytokines TNF-α and IL-10. It was reported that released TNF-α can have a positive feedback on phagocytosis at early stages of infection [212]. Therefore, the increased secretion of TNF-α may be the reason for the enhanced phagocytosis of *C. albicans*.

Moreover, the increased cell surface hydrophobicity resulting from genistein treatment of *C. albicans* points to influences on the cell wall composition. These may affect its interaction with macrophages via either specific receptor-ligand interactions or changes in physicochemical properties, since cell surface hydrophobicity of *C. albicans* cells affects adherence of *C. albicans* to host tissues [213]. However, genistein treatment of *C. albicans* did not influence β-1, 3-glucan exposure so that specific dectin-1 activation cannot be considered to be the reason for the positive effects on the macrophage functions. The respective mechanisms are yet unknown and their elucidation is a topic for further studies.

MAPK kinase cascades are involved in several stress resistance mechanisms: regulation of cell wall biosynthesis, morphogenesis, and virulence [118, 121, 167]. In particular, CEK1-mediated mitogen-activated protein kinase pathway controls *C. albicans* β-glucan exposure and modulates immune responses [214]. Our results showed that the MAPK mutants have a high phagocytotic uptake efficiency compared with the parental strains CAF2-1, which is

related to higher β-glucans exposure. Cek1p, Mkc1p, Hst7p, and Pbs2p are reported to regulate the virulence of *C. albicans* in *in vivo* experiments [167]. We used genistein to pre-treat the mutants *Δhog1* and *Δcek1*, then infected the macrophages. The results showed no influence of genistein on the phagocytosis of the mutants compared with solvent control, suggesting that these pathways are involved in the genistein effect on phagocytosis.

In conclusion, we studied the effects of genistein on the interaction of *C. albicans* with macrophages, using primarily phagocytosis and cytokine production as indicators. Genistein exerted dose- and time-dependent inhibitory effects when macrophages were pre-treated with genistein. However, genistein also showed the ability to modify the cell surface of *C. albicans* so that the cell surface became more hydrophobic and enhanced the activity of murine macrophages.

Thus, our studies point to new pharmacological activities of genistein and alternative strategies for immunostimulation in host-pathogen systems by treating the immune cells as well as the pathogen.

5.5 Conclusions and future directions

Flavonoids belong to a class of phytochemicals with remarkable chemical and biological properties. Since ancient times, humans have exploited the therapeutic properties of medicinal plants, many of which have been found to have flavonoids as their primary active constituent. Isoflavones are a subclass of flavonoinds which is found mainly in soybeans, which are mostly consumed in Asian countries like China and Japan in the regular diet. The target compound genistein is one of the most important isoflavones. Research on the health benefits of genistein over the last century has elucidated the effects as antioxidant, anti-inflammatory, anti-microbial, cardioprotective, and anti-cancer compound.

Fungal infections are a severe medical issue in particular for patients with a compromised immune system. *C. albicans* infections have risen steadily over the past three decades. Approximately 70% of women experience vaginal infections and 20% of them suffer from recurrent infections. In those latter recurrent infections, about half of the patients have four or more episodes per year [215].

The immune system has two principal components: innate and adaptive immunity, which work together to protect the body against infections. The cells of the innate immune system represent the first line of defense in the immunosurveillance network; they directly recognize a wide variety of microorganisms through highly conserved receptors and essential microbial

molecules. Meanwhile, the immune system carries out its important defensive function in intimate and coordinated interactions with the nervous and endocrine systems. Compromised immune system increases risks of opportunistic infections and uncontrolled neoplastic tissue growth, while an over-activated immune system could also lead to inflammation, allergy, and autoimmunity.

Currently, several classes of systemic antifungal compounds are applied in clinical therapy. The application of these antimycotic agents is challenged by the increasing numbers of resistant strains and by severe side effects. Drug resistant strains cause tremendous economic damage and life-threatening diseases in both immunocompromised and immunocompetent individuals.

Thus, there is an increasing need to develop new agents, which utilize new targets against fungal human pathogens, reduce the risk of resistance development and maintain an efficient and equilibrated immune system.

The research undertaken in this thesis was designed to:

- ✧ Identify potent effects of genistein on the properties of the immune cells including anti-inflammatory effects of genistein against stimuli from bacteria and cytokines and elucidate the underlying mechanisms
- ✧ Determine the molecular effects of genistein on the pathogen *C. albicans* focusing on the modulation of the mitochondria function of *C. albicans*
- ✧ Examine the effects of genistein on the interaction of macrophages and *C. albicans*
- ✧ Examine the role of single gene deletions on the interaction of macrophage and *C. albicans* and on the activity of genistein

The elucidation of the mechanisms of action of genistein against *C. albicans* infection listed above is a challenging task. A multitude of chemical and biological properties of genistein may together collaborate in the anti-inflammatory and anti-fungal effects. The findings outlined in this thesis provide the basis for piecing together a mechanism explaining the properties of genistein against *C. albicans* infection.

We have demonstrated that genistein induces a cell cycle arrest in G2/M phase and influences cell proliferation in high concentration (50-100 μM) with long time incubation (24-48 h). Treatment of the macrophages with genistein for 24 or 48 h also led to significant morphological changes, such as elongation of the cells and development of long pseudopodia-like protrusions. By staining the F-actin cytoskeleton, we observed the accumulation of actin-filaments at the edges of the cells.

Genistein influences the function of macrophage challenged by different stimuli such as LPS

and cytokines like IFN-γ. It showed a strong anti-inflammatory effect, while the precise mechanism underlying this inhibition still remains to be further determined. Possible mechanisms include: (i) inhibition of the secretion of NO; (ii) reduction in the secretion of inflammatory cytokines TNF -α, IL-6, and IL-10 protein levels via influencing of their transcription; and (iii) strong regulation of the expression of (a) cytokines IFN-β, IL-10, IL-1α, IL-1β, IL-6, TNF-α, Csf2, and Csf3; (b) chemokines Ccl2 and Cxcl10; (c) transcription factor NF-κB1; (d) IκB-α; and (e) cyclooxygenase 2 (COX-2). It could be due to tyrosine kinase inhibition of Jak2, Syk, and Src and their downstream kinase PI3k-Akt pathway, which leads to the inhibition of the transcription factors NF-κB and AP-1.

Additionally, we investigated whether genistein directly influences the pathogen *C. albicans*. Our results showed that genistein blocked oxygen consumption of *C. albicans* and induced the production of reactive oxygen species which means that genistein could influence the function of mitochondria of *C. albicans*. The respiratory chain structure of *C. albicans* is more complex and flexible than that of mammalian cells. It is not yet fully elucidated. Therefore, this brings a challenge to the work. The site of action by genistein in complex I and the question of whether genistein induces AOX expression are still to be investigated.

We further studied the effect of genistein on the interaction of *C. albicans* with macrophages, using primarily phagocytosis and cytokine production as indicators. After treatment of the macrophages with genistein, the phagocytotic efficiency for *C. albicans* was decreased in a time- and dose-dependent manner. Moreover, the production of cytokines (TNF-α and IL-10) stimulated by *C. albicans* was strongly inhibited by genistein. The inhibition of phagocytosis after extended genistein incubation may be due to indirect effects on the cytoskeleton. On the other hand, treatment of *C. albicans* with genistein enhanced the susceptibility of the pathogen for phagocytosis by the macrophages and increased the production of the cytokines IL-10 and TNF-α. However, genistein did not influence the layered structure of the cell wall but *C. albicans* became more hydrophobic.

All investigations in this study were performed as *in vitro* experiments. It is reported that genistein also shows protection of the actue lung injury *in vivo*. However, little is known about the function of genistein in more complex systems. And the effect of genistein on *C. albicans* infection should be extended to *in vivo* experiments. Moreover, the mode of action of genistein on *C. albicans* still needs to be further investigated by microarray and protein analysis. Moreover, whether there is a direct link between the inhibition of respiration and the modulation of inflammatory responses to *C. albicans* by macrophage or not still needs to be further studied.

The advantage of genistein is that it shows not only anti-fungal but also strong anti-inflammatory effects. It can target pathogens and modulate the immune condition of the body. Our studies pointed to new pharmacological activities of genistein and alternative strategies for immunostimulation in host-pathogen systems by treatment of the pathogens instead of the immune cells. As a future research direction, it will be interesting to modify the structure of genistein by altering its functional groups to enhance the antifungal effect and then further identify the medical potential of the new compounds.

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7 APPENDIX

Description of the genes included in the TLR pathway cluster

Gene Symbol	Gene name	Aliases	Gene bank	Gene function	Average Ct			
					1	2	3	4
CCL2	Chemokine (c-c motif) ligand 2	MCP1,monocyte chemoattractant protein-1	NM-011333	C-C chemokine activity	25.9	21.04	20.75	24.93
Ly96	Lymphocyte antigen 96	ESOP-1, MD-2, MD2, MGC151162	NM-016923	Adaptor protein	28.74	28.31	24.98	27.7
Ly86	Lymphocyte antigen 86	MD-1	NM-010745	Adaptor protein	24.67	23.28	23.95	21.4
Irak2	Interleukin-1 receptor-associated kinase 2	6330415L08Rik, AI649099, IRAK-2, MGC102586	NM-172161	Adaptor protein	26.28	25.62	22.97	26.87
Nfkbib	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta	IKB-beta, IKappaBbeta, IkB, IkBb, MGC36057	NM-010908	Inhibitor of NF-κB	33.05	32.56	28.45	32.69
Rel	Reticuloendotheliosis oncogene	c-Rel	NM-009044	Transcription factor	27.11	25	22.72	25.76
Hmgb1	high-mobility group box 1		NM_002128	Adaptor protein	26.1	26.01	25.5	24.34
TLR2	Toll-like receptor 2		NM-011905	Receptor	24.54	24.46	22.2	24.91
TLR9	Toll-like receptor 9		NM-031178	Receptor	33.32	33.69	29.54	29.9
fos	FBJosteosarcoma oncogene	c-fos	NM-010234	Transcription factor	22.77	24.84	22.7	20.72
CD80	CD80 antigen	Cd28l,Ly-53	NM-009855	TLR signalling	28.3	29.05	28.01	30.6
CD86	Cluster of Differentiation 86	CD28 antigen ligand	NM-006889	TLR signalling	35	32.19	32.73	34.98
Rela	V-rel reticuloendotheliosis viral oncogene homolog A (avian)	p65	NM-009045	Transcription factor	23.97	25.57	23.01	25.31
MEKK1	Mitogen activated protein kinase kinase kinase 1	MAPKKK1,Mekk,MFKK1	NM-011945	Protein-kinase,Ser/Thr (non-receptor)	29.65	30.71	28.26	27.52
Ticam2	Toll-like receptor adaptor molecule 1	TICAM-1, TRIF	NM-174989	Adaptor protein	29.12	28.27	26.31	28.74
Csf2	Colony stimulating factor 2(granulocyte-macrophage)	GM-CSF	NM-009969	Secreted proteins-hematopoeitins	35	27.39	32.08	35
Csf3	Colony stimulating factor 3 (granulocyte)	G-CSF	NM-009971	Secreted proteins-cell proliferation	35	21.92	23.11	35
Cxcl10	Chemokine (C-X-C motif) ligand 10	C7, CRG-2, INP10, IP-10, IP10, Ifi10,	NM-021274	C-C chemokine activity	32.25	25.28	24.95	29.01

		Scyb10, gIP-10, mob-1						
Ifnb1	Interferon beta 1, fibroblast	IFN-beta, IFNB, Ifb	NM-010510	Cytokine-inflammatory response	35	30.17	30.17	35
IL10	Interleukin 10	IL-10,cytokine synthesis inhibitory factor	NM-010548	Cytokine-inflammatory response	31.58	23.85	25.66	28.6
IL1a	Interleukin 1 alpha	IL-1α	NM-010554	Cytokine-inflammatory response	33.6	23.23	26.35	35
IL1b	Interleukin 1 beta	IL-1β	NM-008361	Cytokine-inflammatory response	35	21.86	22.52	31.71
IL6	Interleukin 6	IL-6	NM-031168	Cytokine-inflammatory response	35	30.15	31.73	35
Nfkb1	Nuclear factor of kappa light chain gene enhancer in B-cells 1, p105	NF-kappaB1, p50 subunit of NF-kappaB, p50/105	NM-008689	Transcription regulator	26.2	22.63	23.23	24.83
tnfaip3	Tumor necrosis factor, alpha-induced protein 3	Zinc finger protein A20	NM-009397	Ubiquitin-editing enzyme	27.84	25.99	25.14	28.03
nfkbia	Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	I(Kappa)B(alpha)	NM-010907	Inhibitor of NF-κB	26.76	26.36	25	25.92
peli1	Pellino 1	--	NM-023324	Adaptor protein	26.88	24.96	23.96	26.73
ptgs2	Prostaglandin-endoperoxide synthase 2	COX2,cyclooxygenase 2	NM-011198	Prostaglandin and leukotriene metabolism	28.18	21.77	22	26.58
TLR3	Toll-like receptor 3		NM-126166	Receptor	28.88	26.48	26.2	28.68
TLR7	Toll-like receptor 7		NM-133211	Receptor	26.56	25.19	25.41	24.69
TLR8	Toll-like receptor 8		NM-133212	Receptor	30	29.28	30.52	30.96
TNF	Tumor necrosis factor	DIF, MGC151434, TNF-alpha, TNFSF2, TNFalpha, Tnfa, Tnfsf1a	NM-013693	Cytokine-inflammatory response	25.61	23.1	21.57	24.48

Note: 1: Genistein- treated (50μM) macrophages RAW 264.7; 2: LPS -treated (100 ng/ml) macrophages RAW 264.7; 3: LPS+ Genistein treated macrophages RAW 264.7; 4 : control